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MAINTAINING FOOD SAFETY BY PROTECTING IRRIGATION WATER FROM FECAL CONTAMINATION

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Maintaining Food Safety by Protecting Irrigation Water from Faecal Contamination

Saskatchewan Ministry of Agriculture ADF Project

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Summary

Water quality in Saskatchewan is directly impacted by a variety of human activities. Agricultural and municipal activities can adversely affect water quality if failures in waste management cause faecal material to be transported into aquatic ecosystems. The resultant pollution can cause illnesses and have lethal consequences when enteric pathogens are present in the faecal material. Therefore, our ability to identify and eliminate faecal contamination of water, now and in the future, is essential to reduce the risk of waterborne disease. Microbial source tracking (MST) is a promising recently emerged technology that can identify animal specific sources of faecal pollution. The potential of MST technology is appealing as it could facilitate rational mitigation strategies to prevent future microbial contamination events, particularly in watersheds where faecal contamination may be the result of both municipal and agricultural inputs. This study first developed and validated a MST strategy in the laboratory and subsequently used the Qu'Appelle Valley, between Regina and Craven as a study site to further validate the approach in a Saskatchewan context. The section of the Qu'Appelle River under study is an important source of irrigation water for several vegetable producers who provide fresh produce to southern Saskatchewan consumers, and protection of the irrigation water quality is of critical importance for food safety and maintaing the excellent reputation of Saskatchewan producers as suppliers of high quality produce. Furthermore, the study area was selected due to the influence of both intensive agricultural activities and municipal development on water quality in the region.

A Bacteroidales-based microbial source tracking strategy was selected for development. The results of the project clearly illustrate the success of this method in accurately determining host specific sources of faecal contamination. In particular, markers for the specific detection of human, ruminant, pig, and bovine, faeces have been validated and new markers for horse and Canada Goose faeces have been developed for use in Saskatchewan. These markers provide both sensitive and specific detection of host specific faecal material. The testing of the MST markers in the Qu'Appelle River and Wascana Creek has clearly demonstrated the benefit of using a combined measurement of both alternative and conventional faecal indicators (*Eschericheria coli*) for a better understanding of the sources and severity of faecal contamination events. In addition to the Bacteroidales-based MST approach, a protocol was developed for the detection of antibiotics in water sources influenced by agricultural and urban inputs. The results of this study suggest that detection of particular antibiotics, such as erythromycin may provide additional useful information when measuring water quality and inputs from wastewater systems. In conclusion, the results of this study support the proposal that bacterial source tracking can provide Provincial government agencies and researchers with effective new tools for comprehensive studies to characterize faecal pollution inputs in Saskatchewan watersheds. Ultimately adoption of these tools can help to increase the effectiveness of identifying and remediating faecal pollution in Saskatchewan water sources and ultimately help protect irrigation water quality in the province.

1.0 Introduction

Irrigation, especially in dry climate agriculture, is critical to successful crop production. Frequently, irrigation water is derived from open sources, such as rivers that have the potential to be impacted by faecal pollution. Pollution of irrigation water by faecal material can have lethal consequences, when pathogens, such as enterohemorragic *Eshericheria coli*, *Salmonella* spp., and *Campylobacter* spp. are transmitted into the water along with the deposited faecal material (Sivapalasingam et al., 2004; Steele and Odumeru, 2004; CDC, 2008). Contaminated irrigation water has been suspected in various cases of human infections associated with consumption of fresh or minimally processed fruits and vegetables (Steele and Odumeru 2004). A recent significant example in North America was the 2008 *Salmonella* serotype Saintpaul outbreak in the USA that was traced to peppers irrigated with *Salmonella* contaminated water. The consequence was one of the largest nationwide outbreaks of food-borne illness in the USA (CDC, 2008). These large outbreaks emphasize the importance of efficiently protecting open source irrigation water from faecal pollution and subsequently monitoring the quality of irrigation water routinely. The guidelines regarding microbial water quality for irrigation water vary depending on the regulatory agency. A limit of 1000 faecal coliforms per 100 ml of water has been recommended by the World Health Organization (WHO) for unrestricted irrigation (Shuval 2007), the Canadian Water Quality Guidelines for Irrigation (CWQGI) has recommended more stringent guidelines and suggests a maximum allowable count of 100 faecal coliforms per 100 ml and 1000 total coliforms per 100 ml of irrigation water (Jones and Shortt 2005). The use of faecal coliform monitoring to assess irrigation water quality will remain an important tool in irrigation water quality assurance. However, this tool has significant limitations in helping vegetable producers reduce or eliminate faecal contamination of open sources of irrigation water. Sources of faecal contamination are not indicated when using conventional *E. coli* detection procedures, and source identification can be of great value to a vegetable producer in remediating the contamination. Furthermore, the consistent reports of long term persistence of *E. coli* within aquatic systems may limit the ability of *E. coli* detection in identifying recent faecal contamination events (Van Elsas et al., 2011; Ishii and Sadowsky, 2008). Microbial source tracking (MST) is a promising recently emerged technology (Domingo et al., 2007; Field and Samadpour 2007) that may assist in developing and validating rational mitigation strategies to prevent future microbial contamination of irrigation water sources. Despite the recent growth in MST research, development and validation of these new faecal

indicators are required before MST can be used with confidence to identify and reduce faecal contamination of irrigation water sources. This project sought to determine the potential of a Bacteroidales MST approach for measuring faecal pollution in the Qu'Appelle River, in an area used for irrigation of fresh leafy green produce. Bacteroidales are faecal anaerobes that provide a suitable alternative to coliform bacteria (e.g. *E. coli*) as indicators of faecal contamination in water for a variety of reasons. Bacteroidales are present in faecal material at concentrations 1000-fold greater than coliform species; making them a potentially highly sensitive indicator of faecal contamination (Fiksdal et al. 1985). Additionally, because Bacteroidales are anaerobic, they have a poor survival rate outside of the intestinal environment (Fiksdal et al. 1985) and this is useful in predicting future contamination events since their viable presence in the environment is an unambiguous indicator of ongoing contamination. Bacteroidales MST has only recently been developed and remains to be tested in Saskatchewan for irrigation water protection. Because the Bacteroidales approach is relatively new its efficacy has been investigated at limited locations and little is known regarding the geographical robustness of host specificity within the Bacteroidales. As well, the persistence and transport of Bacteroidales in the environment needs to be vigorously quantified to estimate the spatial and temporal range over which faecal inputs can be detected. Detection of the Bacteroidales host-specific gene markers can be accomplished via conventional end-point polymerase chain reaction (PCR) which provides a qualitative presence or absence result. Quantitative PCR (qPCR) approaches have also been developed which provides a quantitative measure of the abundance of the Bacteroidales gene marker. Both approaches were tested in this study.

Tracking sources of faecal contamination can also involve non-biological approaches, such as determining the presence of particular chemicals linked to specific sources of contamination (Blanch et al., 2006). This study examined the detection of veterinary antimicrobials as a possible indicator for water quality. Earlier work of co-applicant, A. Cessna, has involved the detection of agriculturally related chemicals within watersheds (Kuchta and Cessna, 2009; Cessna et al., 2006). Detection of veterniary antimicrobials may provide an additional tool to determining sources of faecal contamination in agricultural watersheds. Swine, poultry and cattle are increasingly being produced in large confined animal feeding operations. With such production, antimicrobials are administered to prevent disease and to promote growth by increasing the rate of weight gain. Since many antimicrobials are poorly absorbed through the gut, they can be excreted up to 80% or more in the faeces and urine.

Recently, other researchers have shown that antimicrobials used in the livestock industry can be detected in surface waters in Canada (Forrest et al., 2006), the United States (Campagnolo et al., 2002) and Europe (Hirsch et al., 1999). Since faecal contamination frequently enters the Qu' Appelle watershed (Fremaux et al 2009a) it is important to determine if antimicrobials are also entering the watershed. Detection of veterinary antimicrobials would imply that animal waste may be a factor in the faecal contamination and the information, combined with data from the microbial source tracking, can be used to identify the potential sources of contamination.

The study site for the project includes a portion of the Qu'Appelle Valley watershed (52 000km^2). The Qu'Appelle River extends over 400 km from headwaters near Lake Diefenbaker to join the Assiniboine River in Manitoba. More than 95% of the drainage area is composed of agricultural fields (wheat, barley, canola) and cattle pastures, but the Qu'Appelle River also runs through urbanized areas such as the towns of Lumsden and Craven (Hall et al. 1999). A series of seven dams and control structures along the river have created reservoirs such as Buffalo Pound Lake, Pasqua Lake and Katepwa Lake along the Qu'Appelle River valley. The discharge regime of the Qu'Appelle River is thus a mix of the regimes of a typical prairie river and lake drainage, and is modified by water additions from the south Saskatchewan River system at Lake Diefenbaker (Pomeroy et al. 2005). Within the selected study site the Qu'Appelle River is under the influence of Wascana Creek, which receives effluent from the Regina city municipal wastewater treatment plant. The study area also includes locations where water is drawn from the Qu'Appelle River for irrigation of fresh leafy vegetables. Contributing sources of contamination to the Qu'Appelle River include diffuse inputs from wildlife, source specific inputs from livestock operations, rainfall runoff events, as well as effluent discharges of intermittent poor quality and unidentified storm water and/or sanitary sewer discharges of unpredictable nature (Water Pollution Control Branch 1984). The detection of *E. coli* levels above the recommended limit of 100 CFU per 100 mL (Jones and Shortt, 2005) within the study area range from 0% to 100% since sampling began in 2005. The frequent and variable inputs of faecal material into the sampling sites (Fremaux et al., 2009a) confirmed its suitability for field testing of the *Bacteroides* based MST approach. Combined with the long history of consistent land use and descriptions of the hydrology it is an excellent site to study the efficacy of newly developed MST tools.

2.0 Methods

2.1 Study Site and water sample collection:

Sites along the Wascanna Creek and Qu'Appelle river from Regina to Craven were sampled at weekly intervals during the growing seasons of 2008, and 2009. The original proposal was to sample in 2009 and 2010, however due to efficient results obtained in the lab we were able to begin our sampling earlier than planned. Figure 1 provides a schematic of the sampling area. All samples were stored at 4 C and were delivered to the lab for subsequent analysis within 3 hours of collection. Weekly sampling was not conducted in 2010 because the successful large sampling efforts of 2008 and 2009 resulted in sufficiently robust data to evaluate the *Bacteroidales* based markers. Therefore in 2010 we were able to improve upon the original proposal by adding a study on persistence of the Bacteroidales faecal indicator markers (see methods section 2.5 for more detail).

Water samples collected in the field, were processed in the lab for subsequent PCR analysis as follows: Samples were concentrated by filtering 400 ml of water through a 0.45 µm pore size membrane using a filter funnel and vacuum system (Millipore Inc., USA). For each sample, the filter was placed in a sterile 15 ml falcon tube (Fisher Scientific, Ottawa, ON, Canada) containing 5 ml of sterile distilled water. The tubes were thoroughly vortexed and 4 ml of this solution was centrifuged at 11,000 g for 3 min. The pellet was resuspended in 200 µl of sterile distilled water and DNA was extracted from this suspension by using the Power Soil DNA kit (MoBio Laboratories Inc.), according to the manufacturer's instructions.

2.2 Faecal material collection for testing sensitivity and specificity of host specific *Bacteroides* PCR markers:

To determine the specificity and sensitivity of the different *Bacteroides* primer sets (Table 1) designed for detection using conventional PCR, a total of 265 individual fresh faecal samples were aseptically collected from 12 different host groups and 8 human derived raw sewage samples were obtained from 4 localities in Saskatchewan (Prince Albert, Lloydminster, North Battleford and Regina). Individual human specimens (n = 54) were provided by the Disease Control Laboratory of

Saskatchewan. Cow (n = 51), chicken (n = 21) and part of the pig faeces (n = 11) were collected as certainly as possible from separate animals from southern SK farms. Additional faecal samples from pigs (n = 39) were obtained from the Prairie Swine Centre (Saskatoon, SK). Faecal samples from other animal species (n = 80) were taken from the Calgary zoo (Alberta), except the goose samples (n = 20) that were collected from the Wascana Lake (Regina, SK) (Table 2). Samples were transported to the laboratory on ice and kept at 4°C until the time of analysis. Additional samples from similar locations were obtained for testing of the quantitative PCR Bacteroidales markers (Table 5)

DNA was directly extracted from 0.25 g of faecal material or 0.25 ml of raw sewage by using the Power Soil DNA kit (MoBio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Water samples from the Qu'Appelle River were first concentrated by filtering 100 ml of water through a 0.45 µm pore size membrane using a filter funnel and vacuum system (Millipore Inc., USA). For each sample, the filter was placed in a sterile 15 ml falcon tube (Fisher Scientific, Ottawa, ON, Canada) containing 5 ml of sterile distilled water. The tubes were thoroughly vortexed and 2 ml of this solution was centrifuged at 11,000 g for 3 min. The pellet was resuspended in 200 µl of sterile distilled water and DNA was extracted from this suspension by using the Power Soil DNA kit (MoBio Laboratories Inc.), according to the manufacturer's instructions. The DNA was used in the PCR assays as described below in section 2.4.

2.3 Enumeration of *E. coli* by using the Colilert 18/Quanti-Tray system.

The Colilert method was used to process river water samples. Each undiluted or appropriate diluted water sample (100 ml) was added to the Colilert 18 reagent and poured into a 97-well Quanti-Tray system, sealed and incubated at 37°C for 22h according to the manufacturer's instructions (IDEXX). Total coliforms and *E. coli* were simultaneously detected and enumerated by counting the number of yellow wells and yellow/fluorescent wells under UV light, respectively. The most probable number (MPN) could be estimated from a chart provided by the manufacturer. Because *E. coli* is a more specific indicator of faecal contamination (Buckalew et al., 2006), only data observed for these bacteria are considered (results were reported as MPN per 100 mL).

2.4 Conventional PCR and Quantitative PCR procedure for detection of host-specific *Bacteroidales* and bacterial pathogens in the Qu'Appelle Valley

Conventional End Point PCR assays with primers designed for total, human, ruminant and pig-specific *Bacteroides* markers was applied on faecal (including raw sewage) and water samples using primer pairs previously designed by Bernard and Field (2000a, 2000b) (Table 1). All amplification reactions were processed in the MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA), and carried out in a total volume of 25 µl reaction mixture containing 5 µl of template DNA, 2 mmol l^{-1} $MgSO_4$, 0.2 mmol l^{-1} of each deoxynucleoside triphosphate (Invitrogen, Carlsbad, USA), 0.4 µmol l^{-1} of each primer (Sigma, Ontario, Canada), 1X Taq reaction buffer, 3 µg of Bovine Serum Albumine (Sigma, Ontario, Canada) and 1 U of Taq DNA polymerase (UBI, Calgary, Canada). The amplification program used was as follows: 5 min initial denaturation of DNA at 95°C, followed by 30 cycles of 30s denaturation at 94°C, 30s primer annealing at the temperature specific for each primer pair (Table 1), and 1 min extension at 72°C. Amplification was completed by a final extension step at 72°C for 6 min. PCR products were separated by gel electrophoresis on 1.5% agarose and visualized by ethidium bromide staining.

Real-time Quantitative PCR (qPCR) detection of total, human, ruminant and bovine-specific *Bacteroidales* markers was applied on water samples using primer and probe sets previously designed (Table 2). To estimate horse faecal pollution in the water samples, a new primer and probe set was developed from a previously identified horse-specific 16S rRNA *Bacteroidales* sequence. All real-time PCR assays were conducted on an iQ5 Real-Time PCR Detection system (Bio-Rad Laboratories). Annealing temperatures as well as primer and probe concentrations were experimentally optimized to obtain optimal performance for specificity and sensitivity. The reaction mixture contained 4 µl of template DNA, appropriate concentrations of primers and probe (Table 4), 5 µg bovine serum albumin (Promega, WI, USA), 12.5 µl iQ Supermix (Bio-Rad Laboratories) and 25 µl water q.s.p. The amplification program used for the Hum-*Bac* and Rum-*Bac* assays was as follows: 95°C for 6 min, followed by 40 cycles of 95°C for 15 s, 61°C (Hum-*Bac*) or 60°C (Rum-*Bac*) for 15 s and 72°C for 45 s. Amplification program for the All-*Bac*, Hor-*Bac* and Bov-*Bac* assays consists of: 95°C for 6 min, followed by 40 cycles of 95°C for 15 s (Bov-*Bac*), 20 s (Hor-*Bac*) or 30 s (All-*Bac*) and 60°C for 45 s

(All-*Bac* and Hor-*Bac*) or 1 min (Bov-*Bac*). Standard curves for all different qPCR assays were generated using serial dilutions of recombinant plasmid DNA containing known copy numbers of appropriate target genes. The qPCR negative controls (no DNA) as well as plasmid DNA standards were always performed in triplicate and DNA template from water samples were processed in duplicate. Furthermore, a synthetic DNA internal amplification control (IAC) was included in all Bov-*Bac* qPCR reactions in order to confirm that false-negative results due to Taq inhibitors that could be present in the DNA extract did not occur. The IAC used in this study, with the sequence 5'CGGCCAAATACTCCTGATCGTCCTCTAATGGAAAATGGAT-GGTATCTATTACTCATAGGCACTAGGAACAGGCGGCGACGAAACTACAGACAAAATTATCTC AAGGAACGCAACAAGC-3', was slightly different than that described by Shanks et al. (2008). It contained the same primer binding sequences as the bovine-specific *Bacteroidales* DNA target, plus a sequence that hybridized with a probe labelled with the TET fluorophore in order to distinguish amplification of the IAC DNA sequence from that of the target. An IAC concentration of 10^{-10} µM was determined as the most appropriate since it does not compete with the target amplification.

2.5 Persistence of the *Bacteroides markers* in the environment

The persistence study was conducted in the Wascana Creek, upstream of the city of Regina. Three trials were preformed during the summer of 2010, with each trail lasting between fourteen and seventeen days. Two pieces of rebar were placed one meter apart within the flow of the creek. Bottles were secured on wire with plastic ties and positioned half a meter below the surface of the water. HOBO Pendent Data Logger's were attached to the rebar to collect temperature and light data (Onset Computer Corporation, Bourne, MA). In trials one and two duplicate replicates were placed in the creek.

Individual faecal samples, cows, were collected from a local location. Human waste water, primary influent, was collected from the Regina Waste Water Treatment Plant. DNA was extracted from all cow samples to confirm the presence of genetic markers then frozen at -20C. Primary influent was collected three days prior to the start of each trial and stored at -4C. Five grams of individual faecal sample was weighed out and added to 700 ml of sterile water. The contents were homogenized and debris was allowed to settle for 10 minutes. 100ml of supernatant was added to 500ml sterile plastic bottles. Waste water was mixed and 40ml was added to each bottle. In the field 160ml of river water

was aseptically added to each bottle, bringing the final volume to 300ml. Water samples were collected every day for the first 5 days and then twice per week until the host specific *Bacteroidales* markers were below the limit of detection using qPCR. DNA was extracted from the water samples as described in section xx and qPCR reactions were as per described in section 2.1. E. coli enumeration was performed using the Colilert-18/Quanti-Tray system as described in methods section 2.3.

2.6 Detection of agriculturally related antimicrobials in the Qu'Appelle River and Wascana Creek

Solar powered autosamplers were programed to collect weekly composite samples approximately from May to August/September from sample sites along the Qu'Appelle River and Wascana Creek. In 2008 three autosamplers were installed at sites 4, 5, and 6 (figure 1), in 2009 four autosamplers were installed in locations based on results obtained in 2008 at sites 1, 4, 5 and 6. In 2010 weekly grab samples were collected at two sites along the Wascana Creek, upstream (50°23'48.71"N 104°29'9.96"W) and downstream (50°29'3.07"N 104°46'42.13"W) of the Regina wastewater treatment plant.

3.0 Results and Discussion

3.1 Conventional End Point PCR assays for detection of host specific *Bacteroidales* markers in Saskatchewan water sources.

The host specificity data are summarized in table 2. The *Bacteroides-Prevotella* group-specific 16S rRNA marker was present among all the faecal and raw sewage samples tested (Table 2). The detection ability of the PCR assay with the human-specific primer (HF183) was 100% on raw sewage versus 94% (51/54) on human faeces. Similarly, a high detection ability of 98% (119/121) and 100% (50/50) was obtained for the ruminant (CF128) and pig (PF163)-specific primer, respectively. Notably, the CF128 marker was unable to distinguish between domestic (cow) and wild (moose, deer, caribou, bison and wild goat) ruminants confirming that it is a ruminant marker. The HF183 and PF163 primers both exhibited a specificity of 100% suggesting that no cross-amplification with faecal DNA from the other species was observed. By comparison, the specificity of the CF128 primer to differentiate ruminant from the non-ruminant species was 92.8 % (141/152) as the CF128 marker was also detected in 22% (11/50) of the pig faeces. The fact that this primer failed to differentiate between ruminant and

pig (a non-ruminant) faeces was previously reported in Europe (Gawler et al., 2007; Gourmelon et al., 2007). Therefore, detection of the CF128 marker alone indicates possible contamination with ruminant and/or pig faeces. But as appropriately noted by Gourmelon et al. (2007), faecal pollution by pigs can be confirmed by detection of both CF128 and PF163 markers. In addition, the CF128 marker was unable to distinguish between domestic and wild ruminants, and consequently its presence in water does not necessarily indicate faecal contamination from cattle origin. The marker can still be useful in determining cattle contributions to faecal pollution when land use survey data is also considered with the CF128 results.

The detection thresholds of the *Bacteroidales* markers, indicated the assays provide a high level of sensitivity. The detection threshold of the Bac32 marker was identical in all the various sources of faecal material (i.e. detection was possible with a 1×10^{-7} dilution of the fecal sample), whereas it was two orders of magnitude lower in sewage samples (i.e. 1×10^{-5}). Except for the human faeces, the detection threshold of each species-specific Bacteroidales PCR assay was reproducible from one sample to another, and was usually one order of magnitude lower than that obtained for Bac32. In fact, the CF128 and PF163 markers were both detected up to dilution 1×10^{-6} g of each cow and pig fresh faeces, respectively. The HF183 marker was detected up to a dilution of 1×10^{-4} in all the raw sewage samples, but its detection threshold varied from $1 \times 10^{-75}$to 1×10^{-7} depending on the human faecal sample tested. This variance in detection threshold, whereas the threshold of detection of the general Bacteroidales marker Bac32 was similar in the three human faeces, suggests that the prevalence of the Bacteroidales species carrying the HF183 marker may vary between individuals.

By comparison, detection of *E. coli* was up to 10-fold more sensitive than detection of the HF183 in raw sewage. Two previous studies also reported the lower sensitivity of HF183 for detecting sewage than faecal coliforms (Bernhard and Field, 2000a; Bower et al., 2005). A possible explanation would be the uneven distribution and/or degradation of target DNA following the death of most bacteria in the Bacteroidales group due to their lower oxygen tolerance compared to faecal coliforms once released into sewage (Avelar et al., 1998; Savichtcheva and Okabe, 2006) The PF163-specific PCR assay was found consistently as sensitive as *E. coli* in the pig faecal samples tested. Finally, the sensitivity of the CF128 marker was 100-fold higher than that of *E. coli*, which was detected at levels of ca. 10^4 CFU g^{-1} cow faeces. The conventional end-point PCR assays allow sensitive detection of human, ruminant or

pig faecal pollution. The results demonstrated that 1 g of pig or cow faeces could still be detected after dilution in one cubic meter of water using the PF163 and CF128 specific primer, respectively.

Based on the successful laboratory results detection of the markers was tested in the Qu'Appelle sampling sites (Figure 1). All water samples tested positive for the general Bac32 Bacteroidales marker, suggesting that all sites experience some form of faecal pollution. The inability of Bacteroidales to survive under aerobic conditions, especially at high temperatures encountered during summer (Avelar et al., 1998; Seurinck et al., 2005), combined with the recurrent detection of the Bac32 marker suggests that all sampling sites were regularly subjected to contamination by faecal material. However, further research is required to determine the persistence of this marker in environmental locations such as river sediments. In addition, the fact that indigenous uncultured environmental Bacteroidales populations could carry this marker should be considered (Lee et al., 2008). Quantification of the conventional indicator *E. coli* suggests that the degree of exposure to the sources of faecal pollution strongly differed among the sampling locations (Table 3). The detection of E. coli levels above the recommended limit of 100 CFU per 100 mL (Jones and Shortt, 2005) ranged from 100% in water samples collected from site 4 to 0% in those from the C site (Table 3). On multiple occasions, the host origin of a portion of the *Bacteroides* populations could be determined by using the species-specific *Bacteroides* 16s rRNA markers. Whereas the pig-specific *Bacteroides* 16s rRNA marker was not detected in any of the water samples, the HF183 and CF128-specific *Bacteroides* 16s rRNA markers were detected in 41% (29/70) and 14% (10/70) of the water samples taken throughout the Qu'Appelle River on 7 and 4 sampling dates, respectively. Notably, these 2 markers were commonly detected in water sampled from late July till the end of August. As shown in Table 3, the HF183 or CF128-positive water samples were widely dispersed among the sampling sites. Lack of detection of the pig marker is not surprising given the low amount of pig farming activity reported in the Qu'Appelle Valley, and further supports that the CF128 detection is ruminant based. Detection of the host-specific Bacteroidales markers was significantly different in water samples according to their level of *E. coli* ($P < 0.01$, Fisher's exact test). There was a higher prevalence of HF183 and CF128 in water samples with an E. coli level above 100 MPN 100 mL^{-1} than in samples where *E. coli* was below 100 MPN mL^{-1} [68% (17/25) versus 38% (17/45)]. Notably, 8 of the total 10 CF128-positive samples were associated with an E. coli level above 100 MPN 100 mL^{-1}. Both HF183 and CF128 markers were detected at the Wascana, Lumsden or IRPl sites on August 12th when large spikes in *E. coli* numbers.

3.2 Suitability of Quantitative real-time PCR (qPCR) assays for detection of host specific *Bacteroidales* markers in Saskatchewan.

Real-time quantitative PCR methods have been recently developed by several different international groups and used to quantify bovine and human- specific Bacteroidales markers in water samples (Layton et al., 2006; Kildare et al., 2007; Okabe et al., 2007; Reischer et al., 2007; Shanks et al., 2008). This approach may be particularly useful in cases of mixed sources of faecal contamination. Similar to the conventional end point PCR markers (section 3.1) the qPCR makers all displayed excellent host specificity and sensitivity (Table 5). The levels of detection of the Hum-Bac and Rum-Bac markers in individual faecal samples indicates that they occur at a high concentration in individual faeces and to a lower level for the Bov-Bac specific marker (Figures 2-4). Therefore detection of the human and ruminant markers in water following faecal contamination is likely even when the faecal material is significantly diluted following entry into the water source. The bovine marker is present at a lower concentration in cattle faeces relative to the ruminant marker (Figures 2, 3). Therefore detection of the bovine marker in the environment will be more difficult when the cattle faecal contamination occurs at lower levels. However the advantage to this marker is that when it is detected, particularly at high concentration it is likely that a large input of cattle faecal pollution has recently occurred.

Based on the successful laboratory results, detection of the markers was tested in the Qu'Appelle Valley. The advantage of the qPCR approach compared to the conventional end point PCR approach (section 3.1) is clearly illustrated by the data provided in the field study. This approach allowed a quantitative measure of the host-specific marker in 2009 sampling, whereas the conventional PCR assay completed in the Qu'Appelle Valley in 2008 was only qualitative. The results obtained from the host-specific marker detection support observations of land use surrounding the sampling sites. For example, sites 1, 2, 3, and 4 are very close to cattle operations and it was not uncommon to frequently observe cattle in Wascana Creek near the sampling sites. These sites also had the highest concentrations of ruminant marker and were significantly higher compared to the other sites which experience less cattle activity (Figure 5) This provides validation that this marker can be used reliable to identify

ruminant related faecal pollution in sites where land use observations would be less clear. Notably, the concentration of the bovine maker was not significantly higher in sites 1, 2, 3, 4 relative to the other sites. The lack of a correlation between detection of the ruminant DNA marker and bovine DNA marker is further emphasized in figure 6, indicating that the Bacteroidales populations detected by the two markers in cattle faeces are very different. As stated above detection of the bovine marker may be limited due to its lower abundance in cattle faeces and short persistence (see section 3.3). Despite the lack of correlation between the ruminant and bovine markers it is notable that the highest concentrations of the bovine marker did occur in site 3 which is consistently frequented by cattle (Figure 5). Further testing and optimization of the bovine marker is required before this marker can be used with confidence in sites where land use history is not known. Interestingly there is a correlation between the ruminant marker and *E. coli* concentrations in the sites likely impacted by cattle. *E. coli* concentrations were highest in sites 2, 3, and 4. An r^2 value of 0.39 and Pearson p-value of 0.0034 were obtained when comparing the *E. coli* concentration in site 3 with the ruminant marker concentrations. This result is a good example of the benefit of coupling detection of the conventional *E. coli* indicator with the alternative host-specific indicator. In this instance initial *E. coli* enumeration provided a general sense of a faecal contamination problem while the source detection provided information about the possible source of the contamination.

QPCR detections of the human specific marker were consistent across all sampling sites and no particular sites stood out. Due to the consistent low level detection of the human marker we were concerned about its specificity to detect human faecal inputs in the environment. Subsequently, we selected a reference site that we were confident was not be impacted by human faecal pollution. We selected Wascana Lake in Regina for this purpose and 3 sampling sites along the shores of the lake were used. All 3 sites tested negative for the human maker throughout a sampling regime in 2009. This result along with the validated host specificity and lack of long term persistence (section 3.3) provides evidence that detection of the human marker in the Qu'Appelle Valley sites is bona-fide. A consistent low level of human faecal pollution may not be surprising given land use in the area. Several of the sampling sites are surrounded by human habitations that are not serviced for sewage and it is plausible that septic systems are frequently supplying low levels of faecal material into the watershed. These data suggest that further follow-up research on the contributions of human activities (ie sewage treatment practices) in the Qu'Appelle Valley study area are warranted.

3.3 Persistence of qPCR markers in aquatic ecosystems.

An important criteria of an effective faecal indicator is a short environmental persistence, to assure detection and quantification of the indicator relates to recent contamination events and not historical pollution events. The initial data provided by the 2010 studies on Bacteroidales marker persistence are encouraging. The data from two field trials illustrate that the host-specific markers do not exhibit long term persistence in a natural water environment (Figures 9, 10). Notably each host specific marker tested exhibited slightly different persistence times. The bovine specific marker was consistently the most susceptible to degradation with a rapid decay rate, whereas the ruminant marker displayed a slightly longer persistence. In all cases the Bacteroidales markers had a significantly shorter persistence than the conventional *E. coli* marker in the trials and did not persist beyond 12 days. Future persistence experiments are planned and will attempt to better understand the physical, chemical and biological factors that impact persistence. For example water temperature may play an important role in persistence (Balleste and Blanch, 2010) and this will be examined further. Our preliminary data supports this observation as the mean water temperature for the persistence rates with a steeper decay rate (Figure 9) was 2 ºC higher than the mean water temperature for the persistence rates with the slower decay rate (Figure 10).

3.4 Detection of bacterial pathogens *Salmonella* and *Campylobacter* and the relation to the faecal indicators *E. coli* and host-specifc Bacteroidales markers.

The capacity to detect of *Salmonella* and *Campylobacter* during this project was provided through an in-kind contribution from the Canada Research Chair support provided to Dr. Chris Yost. This leveraging of funds successfully increased the scope of the research project. In 2009 extensive sampling for pathogen presence was undertaken at the Qu'Appelle Valley sampling sites. Both *Salmonella* and *Campylobacter* were detected at several sampling sites, while *E. coli* O157:H7 was not detected in any of the samples. An interesting temporal trend was observed for both *Salmonella* and *Campylobacter* presence in the Qu'Appelle samples. Frequency of *Salmonella* detection was highest in the month of August while the frequency of *Campylobacter* detection was highest in the months of May and June (Figure 11). A clear explanation is not readily apparent and further investigation will be

required with additional sampling in subsequent seasons to understand better the seasonal association with pathogen presence in the Qu'Appelle River and Wascana Creek. Notably, the presence of *Campylobacter* did not correlate with levels on any faecal indicators (figure 12), a test with *Salmonella* was not performed due to the low number of *Salmonella* positive samples in the data set. In the previous year, 2008, there was some weak correlation between the conventional PCR ruminant maker and the presence of *Salmonella* (Fremaux et al. 2009b). However this trend was not observed in 2009. The persistence and transport of pathogens may be different than the indicators and therefore further research will be required to determine the ability of the faecal indicators to serve as a proxy for pathogen presence.

3.5 Detection of antibiotics in aquatic ecosystems as a tool for assessing water quality.

Autosamplers were positioned at various sampling sites in the Qu'Appelle study in years 2008 (sites 4, 5, and 6) and 2009 (sites 1, 4, 5, and 6). In 2010 autosamplers were not installed, instead weekly grab samples were obtained at a location downstream of the Regina wastewater treatment plant and upstream of the plant along Wascana Creek. At the start of this project there was no data regarding presence of antibiotic residues in the Qu'Appelle River or Wascana Creek. Several antibiotics were selected for detection based on their use in livestock production and included lincomycin, erythromycin, sulfamethaxine, iso-chloro-tetracycline, tylosin and monensin. Notably only erythromycin and monensin were detected during the sampling periods. The optimized methodology and appropriate controls indicate that lack of detection of the other antibiotics was not due to experimental error. Simply, these antibiotics were not present in the environmental samples at concentrations high enough for detection. Conversely, erythromycin was consistently detected while monensin was infrequently detected in comparison. In 2008, interesting trends were observed across the sampled sites, where site 4 was clearly distinguished from sites 5, and 6 for presence of both erythromycin and monensin. In 2009 a new sampling site was introduced on the Wascana Creek, based on the results from 2008 we targeted an additional sampling site on Wascana Creek. The trend observed in 2008 was not maintained in 2009 as demonstrated in figure 13. Figure 14 indicates that concentrations of the antibiotics rose during June through the beginning of August (Figure 14). An explanation for this is not readily apparent. Future data analysis will investigate hydrological parameters. In both 2008 and 2009 sites 1 and 4 were consistently the highest for erythromycin and in

fact a strong correlation in erythromycin concentration was observed between the sites, suggesting that concentrations observed at site 1 may influence those observed at site 4 (Figure 15). Site 1 is in close proximity to the Regina wastewater treatment plant and maybe be influenced by the plant's effluent. In 2010 samples were taken from Wascana Creek upstream of the wastewater treatment plant and downstream of the plant. Figure 16 indicates that in 2010 a significantly higher concentration of erythromycin was observed in water impacted by wastewater effluent. There was no significant difference between monensin concentrations at the two sites. The data suggests that erythromycin may be a useful marker for contributions of wastewater discharge. These encouraging results merit further investigations to determine prevalence of erythromycin in other wastewater effluents in Saskatchewan as well as to continue monitoring erythromycin concentrations over successive years in Wascana Creek and the Qu'Appelle River, to better understand the concentration fluxes observed between 2008 and 2009. Persistence of eythromycin in aquatic ecosystems is also an area of future study. In conclusion the data supports continued investigations on the use of erythromycin as a potential marker for anthropogenic inputs affecting water quality. Conversely, he data for monensin detection does not support that monensin detection would be a useful discriminatory water quality marker.

4.0 Conclusions and Recommendations

The host specific Bacteroidales markers tested in this study are robust DNA markers capable of sensitive and specific detection of faecal material within a Saskatchewan context. Therefore these markers can be effective at determining both agricultural and human related host-specific sources of faecal contamination within Saskatchewan water sources. These results are in agreement with other recent studies which report that the host specificities of the markers are geographically robust in Europe (Gawler et al., 2007; Gourmelon et al., 2007), Australia (Ahmed et al., 2008), United States (Shanks et al. 2010a), and Asia (Ahmed et al., 2010). The bovine specific marker tested in this study, although specific, is not present at high concentrations in cattle faeces and decays rapidly in the environment. These qualities make it difficult to consistently detect this marker in the environment since its presence is frequently very close to the detection limit of the protocol. Subsequently a recommendation for future study is to investigate other bovine specific markers that have been reported in the literature in the past year (Shanks et al. 2010b). It would also be useful to collect data to test the

supposition that the bovine marker can be effective at identifying severe, recent cattle faecal pollution events by testing for the marker in environments were intensive livestock activity occurs (ILOs).

The persistence data provided by this study further validates the appeal of the *Bacteroidales* based faecal indicator markers. An ideal marker should not persistent in the environment and the preliminary data from this study suggests these markers, unlike *E. coli*, tend to drop below detectable levels <12 days following a contamination event. Although the preliminary data is encouraging further persistence studies are required that include persistence in sediments as well as overwintering persistence. These studies are recommended as future areas of research. Studying persistence of the markers in relation to pathogen persistence is also recommended as a future area of study given that marker presence did not provide a direct linkage to pathogen presence. The causes for notable seasonal abundance of both *Salmonella* (summer/August) and *Campylobacter* (spring) also merit further investigation.

The methodology developed to detect antibiotics related to human and agricultural was successful. Using the developed methodology on sampling of the Qu'Appelle Valley suggests that antibiotic detection, in this case erythromycin, can be added to a water quality monitoring toolbox in both urban and agriculturally influenced watersheds. Furthermore the results from this novel approach highlight the need for expanded research. In particular a more comprehensive analysis of erythromycin prevalence at various sampling sites through out Saskatchewan and a study on its persistence in aquatic ecosystems are warranted.

The *Bacteroidales* MST approach has been demonstrated to be a reliable indicator of host specific faecal contributions in a prairie watershed that is important for irrigation of fresh produce. The data from this study suggests remediation of bovine faecal pollution at particular sites in Wascana Creek is appropriate to protect the water quality. An important future study would be to re-asses the source tracking markers following a remediation strategy to illustrate the effectiveness of the host-specific remediation approach. Ultimately, it is hoped that these types of future studies will showcase the usefulness of *Bacteroides* MST approaches and lead to the incorporation of MST technologies in future policy development for protecting irrigation water in Saskatchewan and Canada. Our ability to

identify and eliminate faecal contamination in irrigation water, now and in the future, is essential to ensure that fresh produce remains free of microbial threats (Solomon, 2003; Byrne et al., 2006).

5.0 Acknowledgements

Support from the Saskatchewan Ministry of Agriculture Agriculture Development Fund has been clearly acknowledged in all public and scientific venues where results from this project were presented. In particular the publications resulting from this research are in high impact journals. Water Research is ranked as the top journal in Water Resources by Journal Citation Reports (2010) and has an impact factor of 4.355. Applied and Environmental Microbiology is the number one cited journal in Microbiology and has an impact factor of 3.686. Future publications that will acknowledge the ADF funding will also be targeted to high impact high readership journals.

The results from this work have also been presented at international conferences and workshops in France thereby further increasing the international exposure of the Saskatchewan Agriculture and Food Agriculture Development Fund.

The results have also been presented in seminars focused specific for irrigation users. I have presented at the Saskatchewan Vegetable Growers Association Annual General Meeting on several occasions and acknowledged the generous support from the Agriculture Development Fund. Furthermore participating in these seminars has allowed me to transfer research knowledge to end users who can benefit from the research.

6.0 Literature Cited

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7.0 Other

7.1 Papers Published:

*Fremaux, B., Boa, T., Yost, C.K. 2010. Quantitative real-time PCR assays for sensitive detection of Canada Goose-specific faecal pollution in water sources. Applied and Environmental Microbiology. 76: 4886-4889.

**although this paper was not a direct deliverable in the ADF grant the knowledge generated during the ADF work greatly facillitated the completion of development of a goose marker and consequently the support of ADF was acknowledged.*

Fremaux, B., Gritzfeld, J., Boa, T., Yost, C.K. 2009. Evaluation of host-specific Bacteroidales 16S rRNA gene markers as a complementary tool for detecting faecal pollution in a prairie watershed. Water Research. 43: 4838-4849

7.2 Papers in preperation:

Fremaux, B., Boa, T., Yost, C.K. Quantitative detection of host-specific Bacteroidales gene markers and their persistence in a prairie watershed. *to be submitted to Environmental Science and Technology impact factor 4.630*

Yost, C.K., Baily, J., Boat, T., Cessena, A. Detection of antibiotics in a mixed agriculture and urban prairie watershed and use as an indicator of water quality. *to be submitted to Water Air and Soil Pollution impact factor 1.676*

7.3 Invited Talks:

Yost, C.K. Protecting water from microbial threats through the development of new faecal pollution indicators. Department of Biology Winter Seminar Series, University of Regina, Regina, SK. January 14, 2011.

Yost, C.K., Fremaux, B., Boa T. The development and application of microbial source tracking technologies in Canada. conference. State of Progress on Microbial Source Tracking (MST) for the Identification of Faecal Pollution. Brest, France. October 29, 2010.

Yost, C.K., Fremaux, B., Boa T. Protecting irrigation water from microbial threats. Saskatchewan Vegetable Growers Annual Conference. Saskatoon, Saskatchewan. November 12-14, 2009.

Freamux, B., Yost, C.K. Detecting sources of faecal contamination in a Saskatchewan watershed. Franco-Canadian Workshop on methods to detect and assess faecal pollution in the environment. University of Lyon, Lyon France. November 1-4, 2009.

Yost, C.K., Fremaux, B., Gritzfeld, J. Ensuring food safety by improving the microbial quality of irrigation water: a study of the Qu'Appelle River in Southern Saskatchewan. 6th International Symposium on Public Health and the Agricultural Rural Ecosystem. Saskatoon, SK. October 19-23, 2008.

Yost, C.K., Fremaux, B., Gritzfeld, J. Protecting the safety of fresh produce by improving the microbial quality of irrigation water: A study of the Qu'Appelle River in Southern Saskatchewan. 58th Annual Meeting for the Canadian Society of Microbiologists. University of Calgary, Calgary, AB. June 9-12, 2008.

7.4 Posters:

Yost, C.K, Fremaux, B., Boa, T., Baily, J., and Cessna, A. Assessing water quality in agriculturally influenced watersheds using *Bacteroidales*-based microbial source tracking and detection of antibiotics. 59th Annual Conference of the Canadian Society of Microbiologists. Concordia University, Montreal, Quebec. June 15-18, 2009.

Fremaux, B., Gritzfeld, J.G., Boa, T.B., Chaykowski, A.C., Kasichayanula, S.K., Braul, L.B., and Yost, C.K. Improving the microbial quality of irrigation water in

Saskatchewan using monitoring and microbial source tracking. 58th Annual Meeting for the Canadian Society of Microbiologists. University of Calgary, Calgary, AB. June 9-12, 2008.

7.5 Workshops:

I participated in a workshop held at the University of Lyon in Lyon France November 1-4, 2009. The purpose of the workshop was to discuss research approaches on detecting and assessing faecal pollution in the the environment and to formulate new collaborations with French researchers and my lab.

A workshop was hosted by the Yost lab and held at the University of Regina on September 21-22, 2010. The workshop brought together Canadian researchers who are experts on microbial source tracking and included both academia and government agencies. In addition a collaborator from IFREMER France presented the keynote, this collaboration was formed following the workshop in Lyon, France, 2009. The program is presented below.

September 21

11:30 am Arrival and Check-in

12:00 pm Lunch and workshop overview

1:00 pm Keynote speaker: Michèle Gourmelon, IFREMER, France: "Development of Bacteroidales markers to identify the origin of the faecal pollution in coastal areas in France"

Different Source tracking markers

2:00 pm Richard Villemur, INRS-Institut Armand-Frappier: Mitochondrial DNA as a Tracer for Faecal Contamination Source Tracking. A case study: The L'Assomption river Watershed

2:30 pm Norman Neumann, University of Alberta/Alberta Health Services: "Source tracking faecal pollution in water using parasites and viruses"

3:00 pm Tom Edge, Environment Canada: "From library-dependent to library-independent MST approaches in studying beach closures around the Great Lakes"

3:30 pm Luke Mason, National Research Council: Technical approaches in Mitochondrial Source Tracking

4:00 pm return to hotel

6:00 pm dinner and discussions

September 22nd

Examples of current Source Tracking Approaches with reference to pathogens

9:30 am Kari Dunfield, University of Guelph: "Effect of tillage and timing of liquid manure application on the transport of N, P and bacteria to surface and subsurface waters"

10:00 am Vic Gannon, Public Health Agency of Canada- "Molecular subtypes of *Campylobacter* spp., *Salmonella enterica*, and *Escherichia coli* O157:H7 isolated from faecal and surface water samples in the Oldman River watershed, Alberta, Canada"

10:30 break

11:00 am Chris Yost, University of Regina: "Detection of Bacteroidales based markers in the Qu'Appelle Valley watershed"

11:30 am Rob Jamieson, Dalhousie University: "Bacterial transport and microbial source tracking investigations within the Thomas Brook Watershed"

12:00 pm lunch

1:00-4:00 pm Discussion on implementation of collaborative projects.

State of Science and Readiness for Widespread Application, some suggested points for discussion:

1. What are the necessary research endeavours required to move forward MST to wider application and into policy development?
2. Opportunities for inter-lab method optimization/validation
3. Which MST markers represent suitable options for an inter-lab study
4. Possible funding sources to pursue for collaborations
5. other points to increase implementation of MST in policy development?-- ie linking source tracking to agricultural BMP (best management practices) studies?

4:00 pm closing remarks and conclusion of meeting.

Table 1: PCR primer sequences for host specific Bacteroidales 16S rRNA gene markers used for conventional PCR assays.

Name	Primer Sequence	Target	Anneal °C	Amplicon Size	Reference
Bac32F	AACGCTAGCTACAGGCTT	*Bacteroides-Prevotella*	58	676	Bernhard and Field (2000b)
Bac708R	CAATCGGAGTTCTTCGTG	*Bacteroides-Prevotella*			
CF128F	CCAACYTTCCCGWTACTC	Ruminant *Bacteroides* species 16S rRNA gene	53	580	Bernhard and Field (2000a)
HF183F	ATCATGAGTTCACATGTCCG	Human *Bacteroides* species 16S rRNA gene	59	525	Bernhard and Field (2000a)
PF163F	GCGGATTAATACCGTATGA	Pig *Bacteroides* species 16S rRNA gene	57.4	563	Dick et al. (2005)

Table 2: Detection of the host-specific *Bacteroides* markers in different species present in Saskatchewan, Canada using conventional PCR.

Host groups	No samples	Positive *Bacteroides* markers detection			
		HF183F	CF128F	PF163F	Bac32F
Humans					
Individual	54	51	0	0	54
Raw sewage	8	8	0	0	8
Total human samples	62	59	0	0	62
Animals					
Cow	51	0	49	0	51
Pig	50	0	11	50	50
Chicken	20	0	0	0	20
Goose	20	0	0	0	20
Moose	10	0	10	0	10
Deer, white tailed	10	0	10	0	10
Deer, mule	10	0	10	0	10
Deer, Fallow	10	0	10	0	10
Caribou	10	0	10	0	10
Bison	10	0	10	0	10
Goat	10	0	10	0	10
Total animal samples	211	0	130	50	211

Table 3: Levels of *E. coli,* and incidence of the host-specific *Bacteroides* 16s rRNA markers and bacterial enteric pathogens in the different sites sampled weekly along the Qu'Appelle River between June and September 2008

Sites [a]	Min-Max *E. coli* values (MPN 100ml^{-1})	Mean *E. coli* amount [b] (MPN 100ml^{-1})	Samples > limit (n/N)[c]	Incidence of HF183 positive	Incidence of CF128 positive
C	16-62	38	0/14 (0%)	7/14 (50%)	1/14 (7%)
4	164-1673	414	14/14 (100%)	6/14 (43%)	3/14 (21%)
L	26-2275	270	5/14 (36%)	5/14 (36%)	2/14 (14%)
5	25-2131	255	5/14 (36%)	5/14 (36%)	3/14 (21%)
6	10-183	40	1/14 (7%)	6/14 (43%)	1/14 (7%)

MPN= most probable number. We did not detect the presence of the swine specific marker in any of the samples and therefore it is not included on the table.

[a] Site numbers correspond to figure 1

[b] The mean total amount of *E. coli* is calculated from the addition of the values obtained during the irrigation season (June-August)

[c] n, number of samples with an *E. coli* level > 100 MPN 100 mL^{-1}; N, number of total samples tested for the corresponding period

Table 4: PCR primer and probe sequences used to detect host specific *Bacteroidales* gene markers using a Quantitative PCR approach.

Name (target)	Primer/ probe name	Sequence (5'-3')	Amplicon size (bp)	Annealing temp (°C)	Reference
All-*Bac* (*Bacteroidales*)	AllBac296f	GAGAGGAAGGTCCCCCAC	106	60	Layton et al. (2007)
	AllBac412r	CGCTACTTGGCTGGTTCAG			
	AllBac375Bhqr	FAM-CCATTGACCAATATTCCTCACTGCTGCCT-BHQ1			
Hum-*Bac* (Human)	BacH_f	CTTGGCCAGCCTTCTGAAAG	93	61	Reicher et al. (2007)
	BacH_r	CCCCATCGTCTACCGAAAATAC			
	Probe BacH_pC	FAM-TCATGATCCCATCCTG-NFQ-MGB			
	Probe BacH_pT	FAM-TCATGATGCCATCTTG-NFQ-MGB			
Rum-*Bac* (Ruminant)	BacR_f	GCGTATCCAACCTTCCCG	118	60	Reischer et al. (2006)
	BacR_r	CATCCCCATCCGTTACCG			
	BacR_p	FAM-CTTCCGAAAGGGAGATT-NFQ-MGB			
Bov-*Bac* (Bovine)	CowM2F	CGGCCAAATACTCCTGATCGT	92	60	Shanks et al. (2008)
	CowM2R	GCTTGTTGCGTTCCTTGAGATAAT			
	Probe	FAM-AGGCACCTATGTCCTTTACCTCATCAACT ACAGACA-BHQ1			
	IAC-probe	TET-TAGGAACAGGCGGCGACGA-BHQ1			
Hor-*Bac* (Horse)	HorF	GCCAGCCGTAAAATAGTCGG	96	60	This study
	HorR	CAATCGGAGTTCTTCGTGATATCTA			
	HorPr	FAM-AACCCGATCCCGCGGTTGGAA-BHQ1			

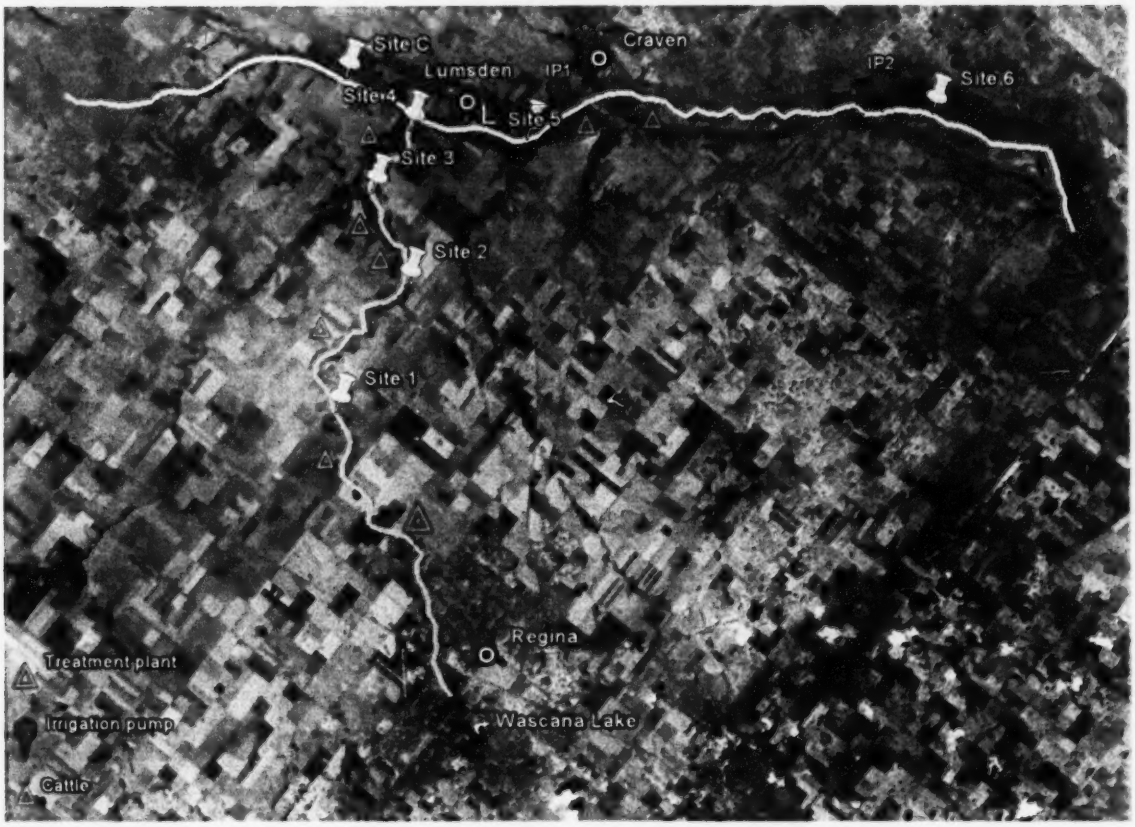
Table 5: Detection of the host-specific *Bacteroides* markers in different species present in Saskatchewan, Canada using the quantitative PCR markers.

Host group	**N° samples**	**Detection of the *Bacteroidales* markers via QPCR**				
		Hum-*Bac*	**Rum-*Bac***	**Bov-*Bac***	**Hor-*Bac***	**All-*Bac***
Humans						
Individual	25	22	0	0	0	25
Raw sewage	6	6	0	0	0	6
Total human samples	31	28	0	0	0	31
Animals						
Cow	41	0	37	36	0	41
Pig	48	0	2	0	0	48
Chicken	34	0	0	0	0	34
Canada Geese	101	0	0	0	0	95
Gull	16	0	0	0	0	14
Pigeon	25	0	0	0	0	22
Duck	10	0	0	0	0	10
Swan	1	0	0	0	0	1
Moose	10	0	10	0	0	10
Deer, white tailed	10	0	10	0	0	10
Deer, mule	10	0	10	0	0	10
Deer, Fallow	10	0	10	0	0	10
Caribou	10	0	10	0	0	10
Bison	10	0	10	0	0	10
Goat	10	0	10	0	0	10
Horse	15	0	0	0	14	15
Total animal samples	361	0	109	36	14	350

Table 6: Levels of *E. coli* and the *Bacteroidales* 16S rRNA markers in the different sites sampled weekly along the Qu'Appelle River during the studying period

Site	*E. coli*			AllBac		Ruminant		Bovine		Human		Horse
	Samples > limit (n/N) (%)	*Mean (MPN 100ml^{-1})*	*Min-Max (MPN 100 ml^{-1})*	*Marker-positive samples (n/N) (%)*	*Mean (Log$_{10}$ copy number 100 ml^{-1})*	*Marker-positive samples (n/N) (%)*	*Mean (Log$_{10}$ copy number 100 ml^{-1})*	*Marker-positive samples (n/N) (%)*	*Mean (Log$_{10}$ copy number 100 ml^{-1})*	*Marker-positive samples (n/N) (%)*	*Mean (Log$_{10}$ copy number 100 ml^{-1})*	*Marker-positive samples (n/N) (%)*
1	10/17 (59)	167	26-637	17/17 (100)	6.2	17/17 (100)	4.3	15/17 (88)	2.9	17/17 (100)	4.1	0/17 (0)
2	15/20 (75)	299	14-794	20/20 (100)	6.3	19/20 (95)	4.3	18/20 (90)	5	20/20 (100)	4	0/20 (0)
3	17/20 (85)	421	22-1072	20/20 (100)	6.5	20/20 (100)	4.6	15/20 (75)	4.7	19/20 95)	4.2	0/20 (0)
4	19/22 (86)	291	18-794	22/22 (100)	6	20/22 (91)	3.9	16/22 (73)	2.9	22/22 (100)	4.3	0/22 (0)
5	11/22 (50)	144	10-733	22/22 (100)	5.8	19/22 (86)	3.8	19/22 (86)	3.1	20/22 (91)	4.1	0/22 (0)
6	1/22 (4.5)	44	13-220	22/22 (100)	6.2	16/22 (73)	3.4	12/22 (55)	3	20/22 (91)	3.8	0/22 (0)
C	3/22 (1.4)	48	5-150	22/22 (100)	5.8	15/22 (68)	3.1	17/22 (77)	3	20/22 (91)	3.7	0/22 (0)

Figure 1: Location of sampling sites along the Qu'Appelle River and Wascana Creek.



Aerial view of the different sampling sites (from site 1 to site 6 and site C) along the Qu'Appelle River. In 2008 a site within Lumsden was also sampled, indicated by "L"The arrows indicate the direction of flow. Potential anthropogenic inputs are also indicated on the figure (Aerial photograph courtesy of http://www.Earth.Google.com). The treatment plant is the City of Regina sewage treatment plant.

Figure 2. Box plot representation of concentration of Ruminant qPCR marker (Rum-Bac) in individual cattle faecal samples.

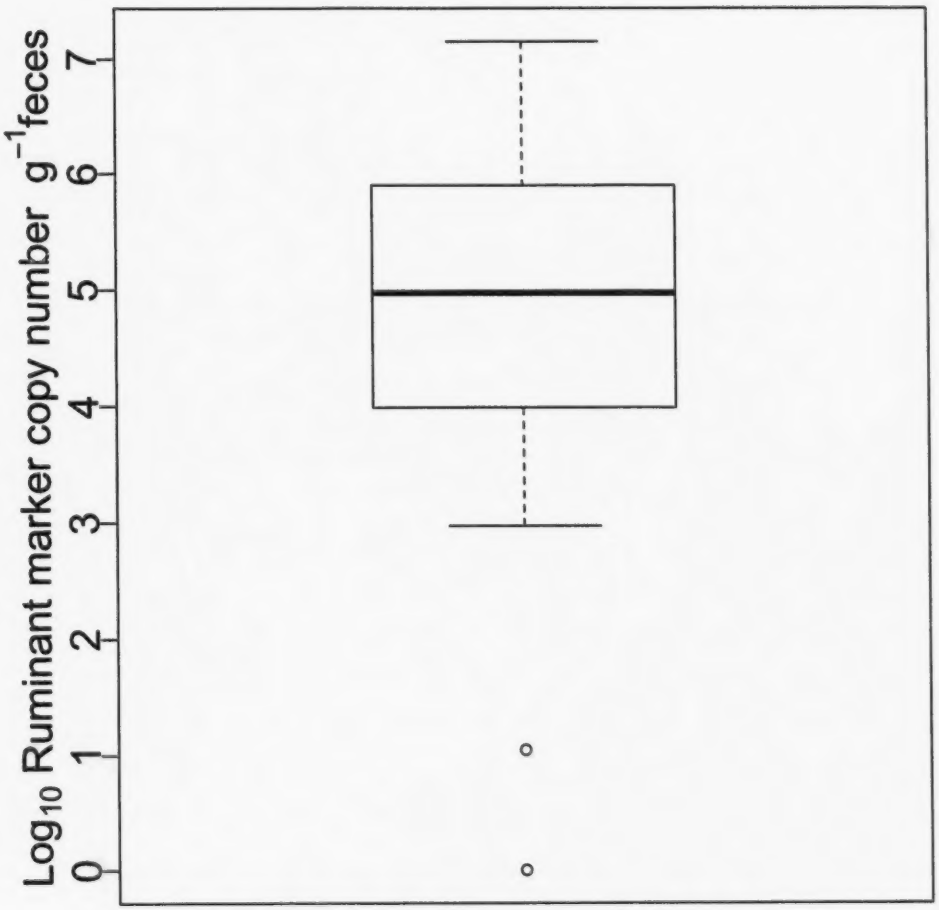


Figure 3. Box plot representation of concentration of Bovine specific qPCR marker (Bov-Bac) in individual cattle faecal samples

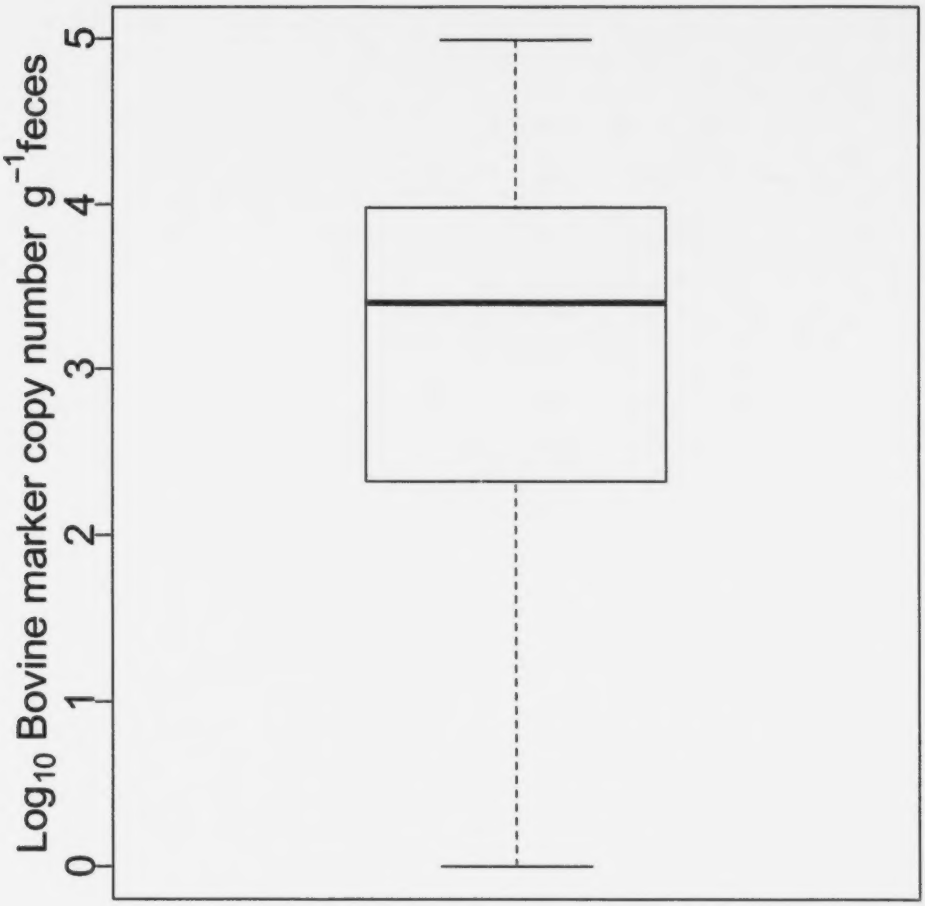


Figure 4. Box plot representation of concentration of Human specific qPCR marker (Hum-Bac) in individual human faecal samples

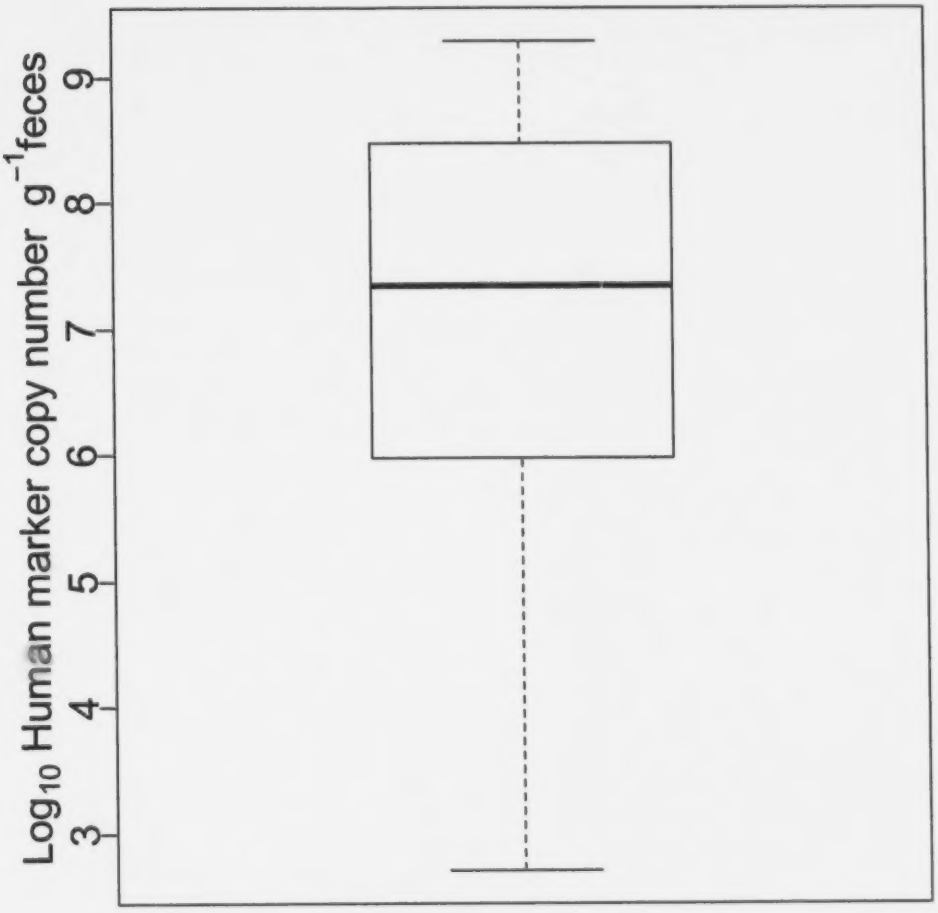
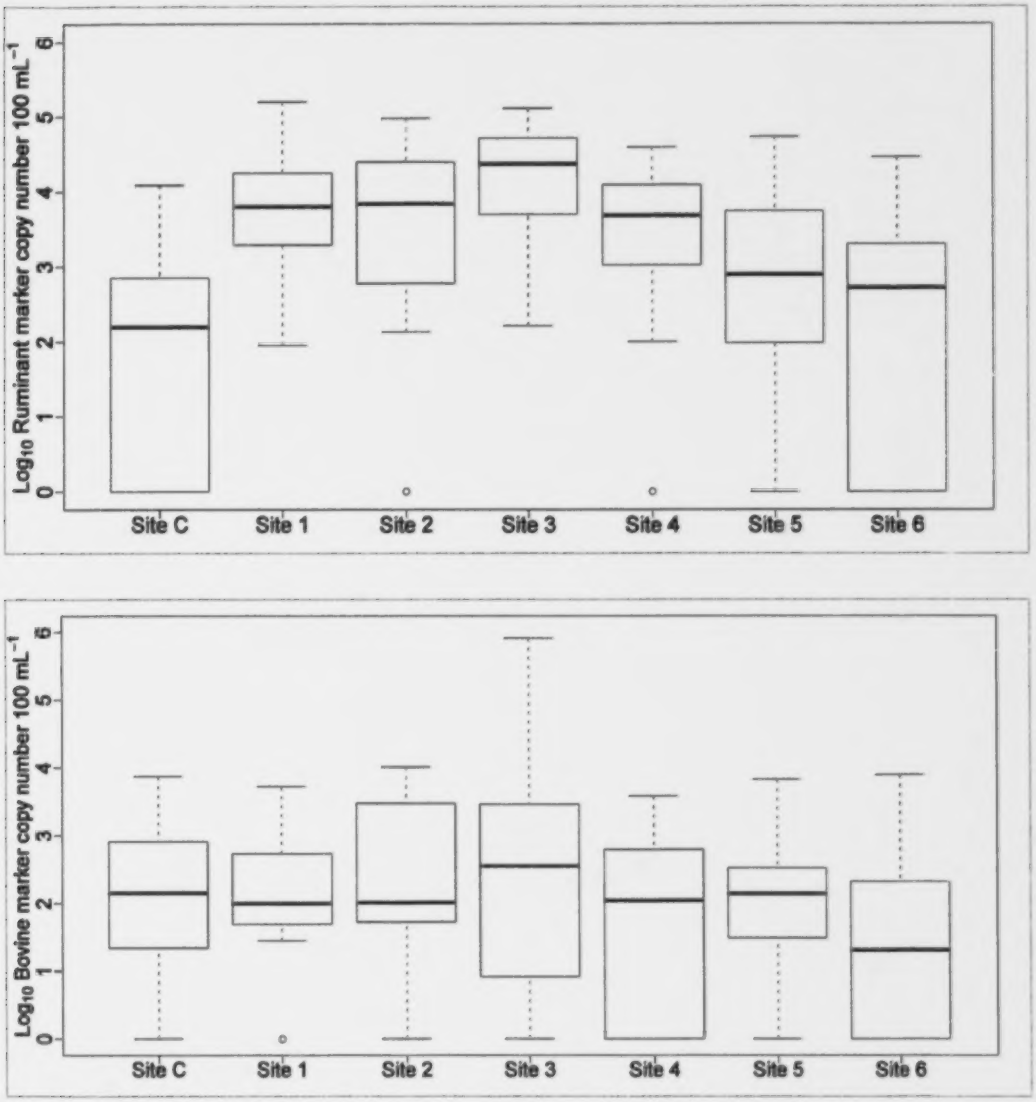
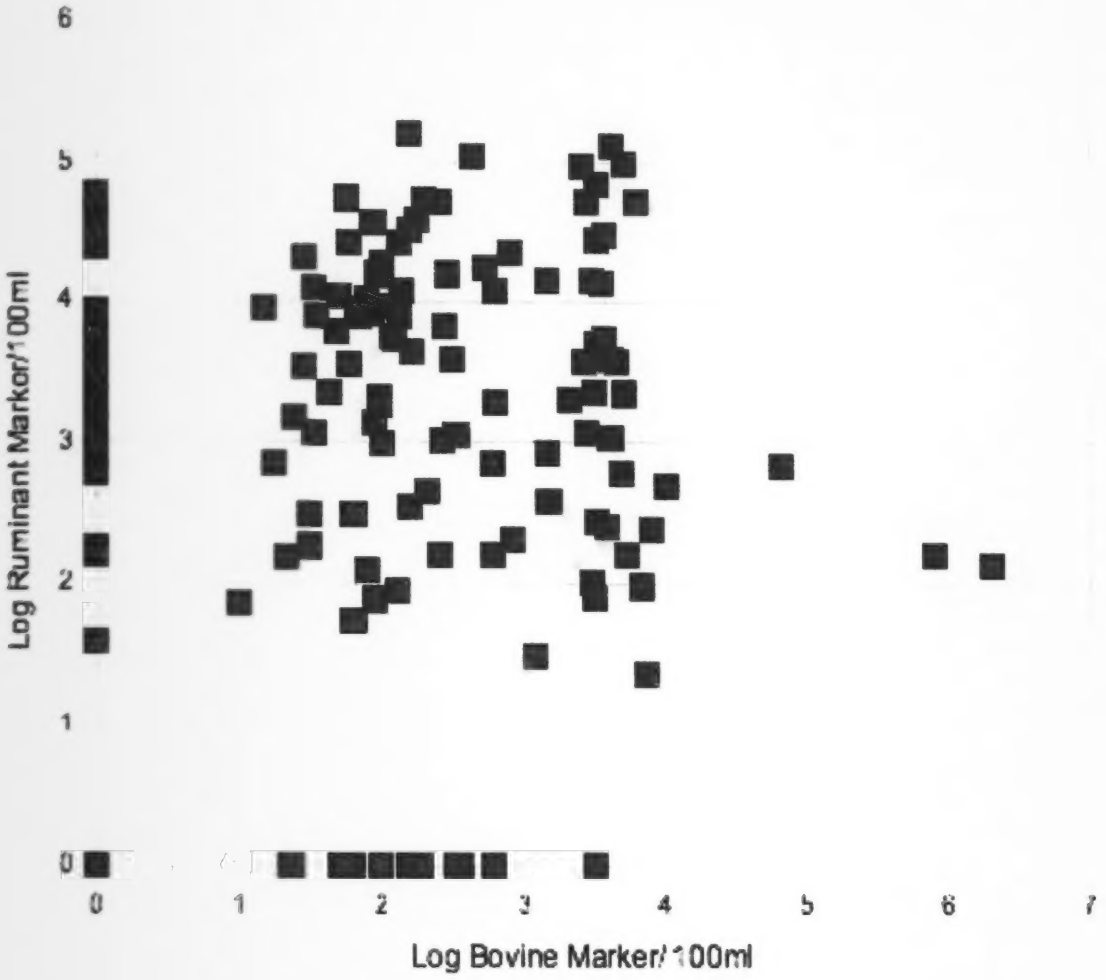


Figure 5. Quantitative detection of the ruminant and bovine specific markers using qPCR during the 2009 sampling, May-September.



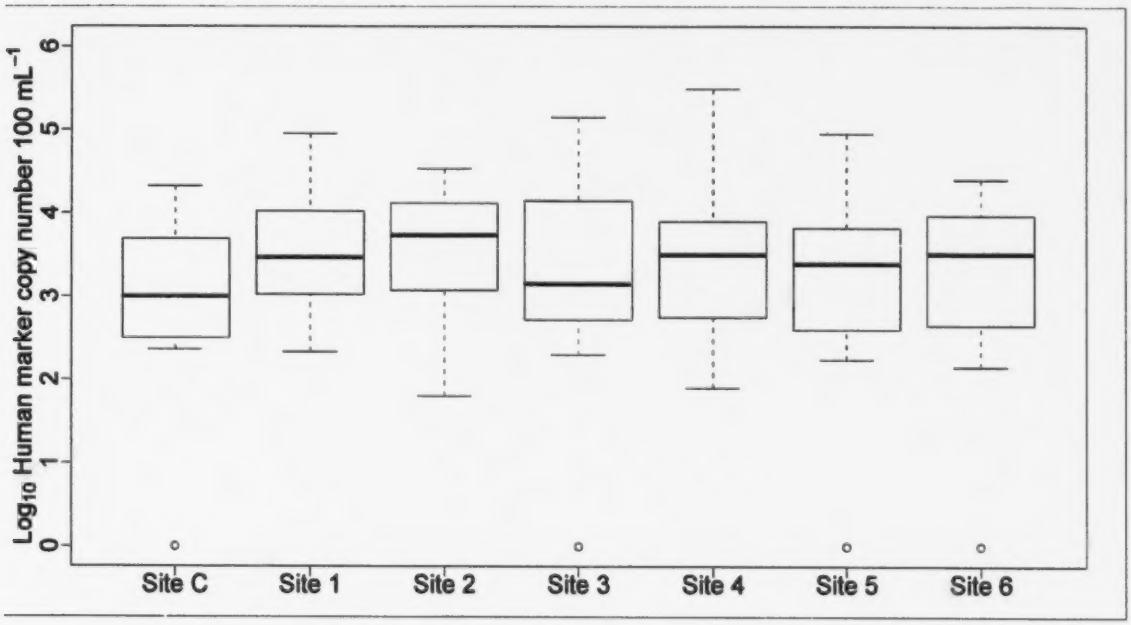
Top panel is the ruminant marker, bottom panel is the bovine marker. Sites 1 to 4 are similar and distinct from sites C, 5 and 6 in the ruminant maker (Kruskal-Wallis, $P>0.0001$). No differences in values were observed for the bovine marker (Kruskal-Wallis, $P>0.05$).

Figure 6. Detection of the ruminant specific maker (Rum-Bac) and its relation to detection of the bovine specific marker (Bov-Bac) during the 2009 sampling season.



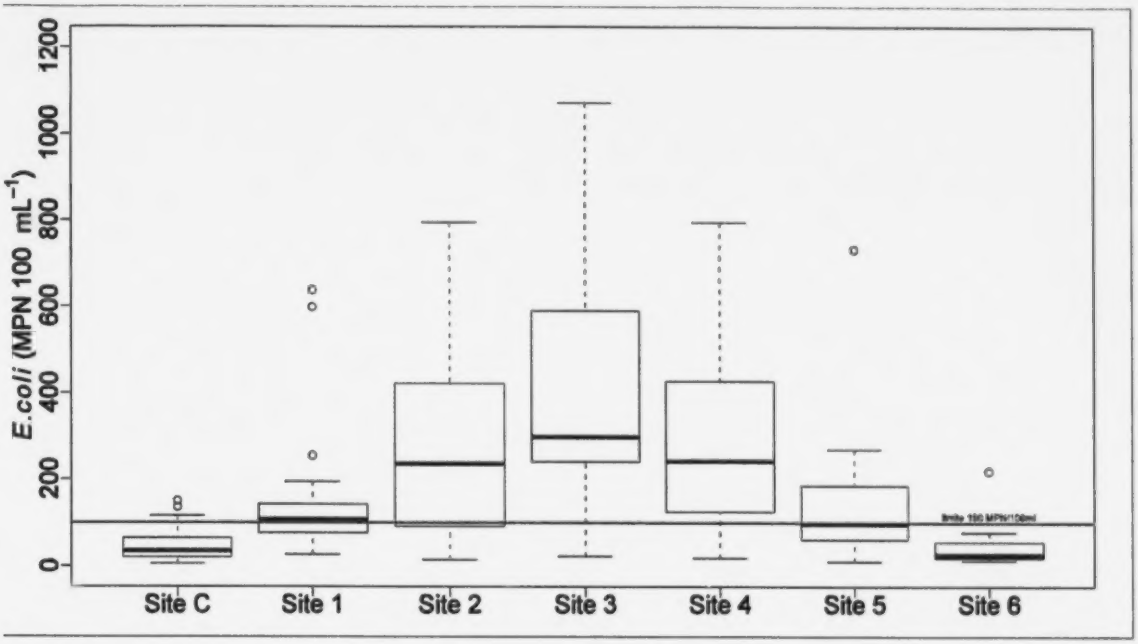
The regression analysis indicates no correlation between the two markers.

Figure 7. Quantitative detection of the human specific marker using qPCR during the 2009 sampling, May-September.



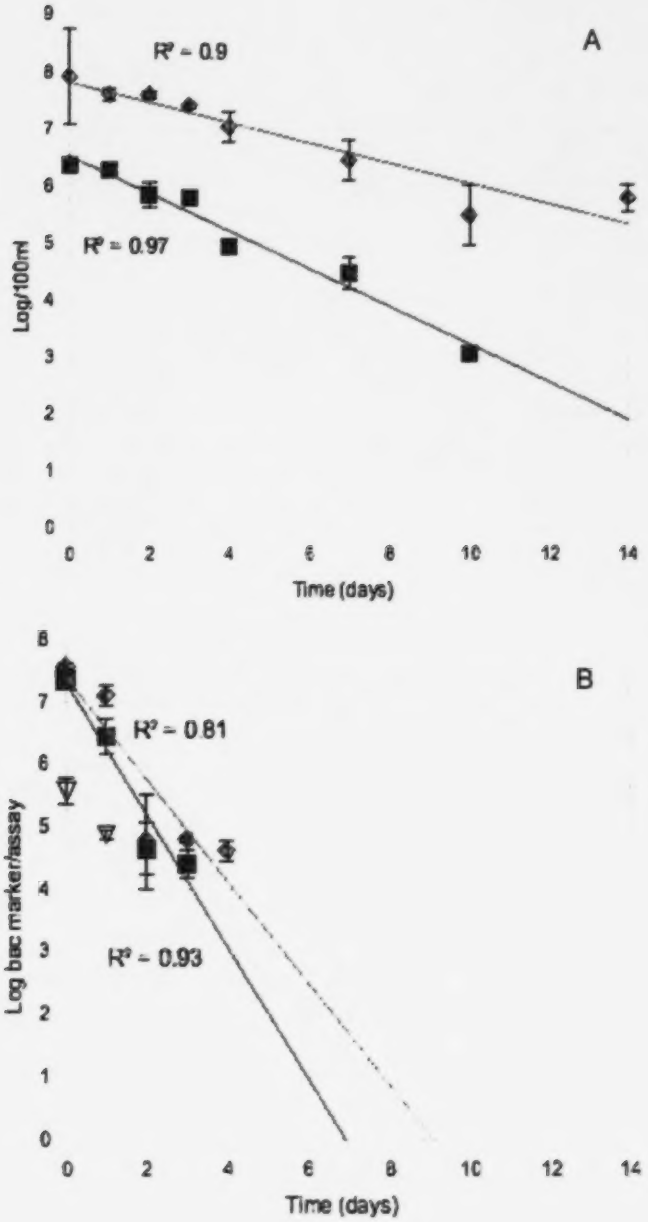
No significant difference in mean concentrations (Kruskal-Wallis, P>0.5). 3 reference sites in Wascana Lake tested negative during weekly sampling in Fall 2009.

Figure 8. Enumeration of *E. coli* during the 2009 sampling season using the Collilert enumeration system.



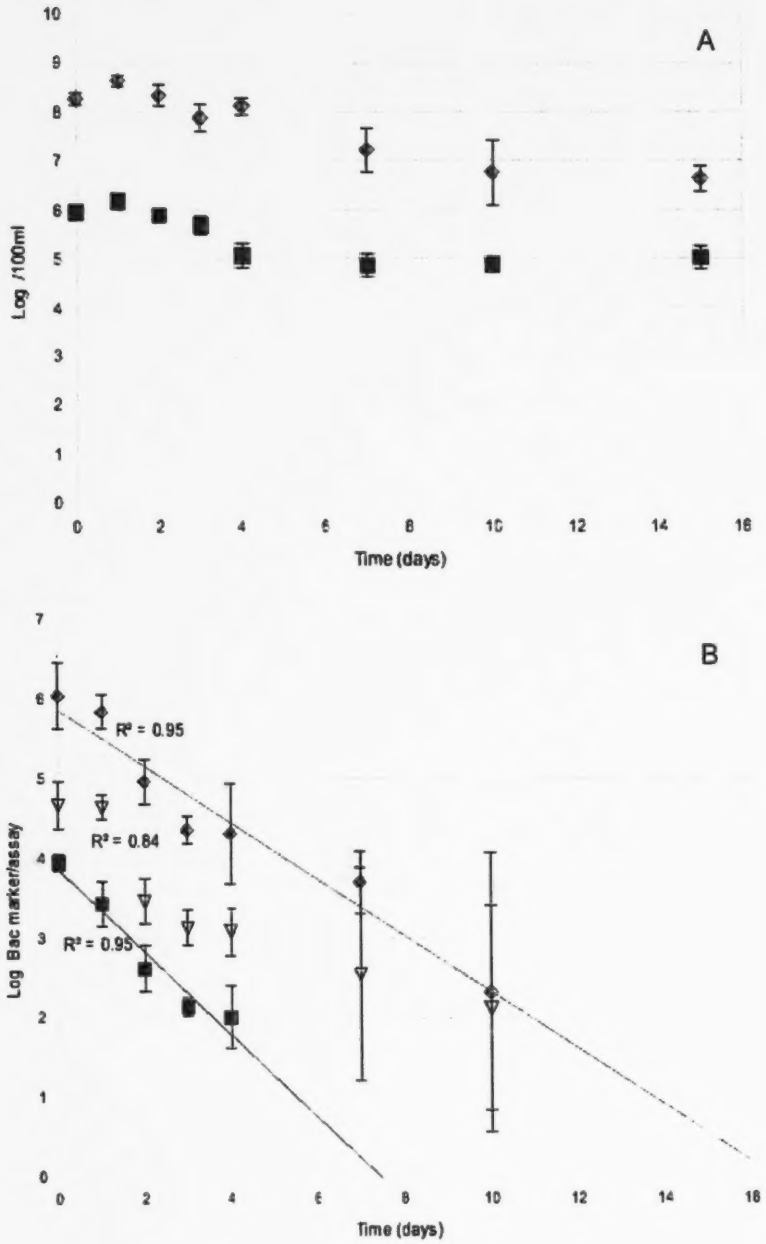
The red line indicates the value of 100 *E. coli* CFU 100 ml-1, which is the maximum allowable limit suggested for irrigation water use as proposed by the Canadian Council of Ministers of the Environment. Sites 5 and 6 are close to irrigation water pumps.

Figure 9. Persistence trial and rates of decay of Bacteroidales DNA markers and *E. coli* of conducted from July 12 to July 26, 2010, in Wascana Creek.



Panel A: red diamond is All-Bac maker, blue square is *E. coli*. Panel B: limit of detection is log 2/assay, the blue square is Hum-Bac marker decay, the yellow triangle is Bov-Bac maker decay, the red diamond is Rum-Bac marker decay

Figure 10. Persistence trial and rates of decay of Bacteroidales DNA markers and *E. coli* of conducted from August 9 to August 24, 2010, in Wascana Creek.



Panel A: red diamond is All-Bac maker, blue square is *E. coli*. Panel B: limit of detection is log 2/assay, the blue square is Bov-Bac marker decay, the yellow triangle is Hum-Bac maker decay, the red diamond is Rum-Bac marker decay.

Figure 11. Detection of *Campylobacter* and *Salmonella* in the Qu'Appelle River and Wascana Creek as a function of month.

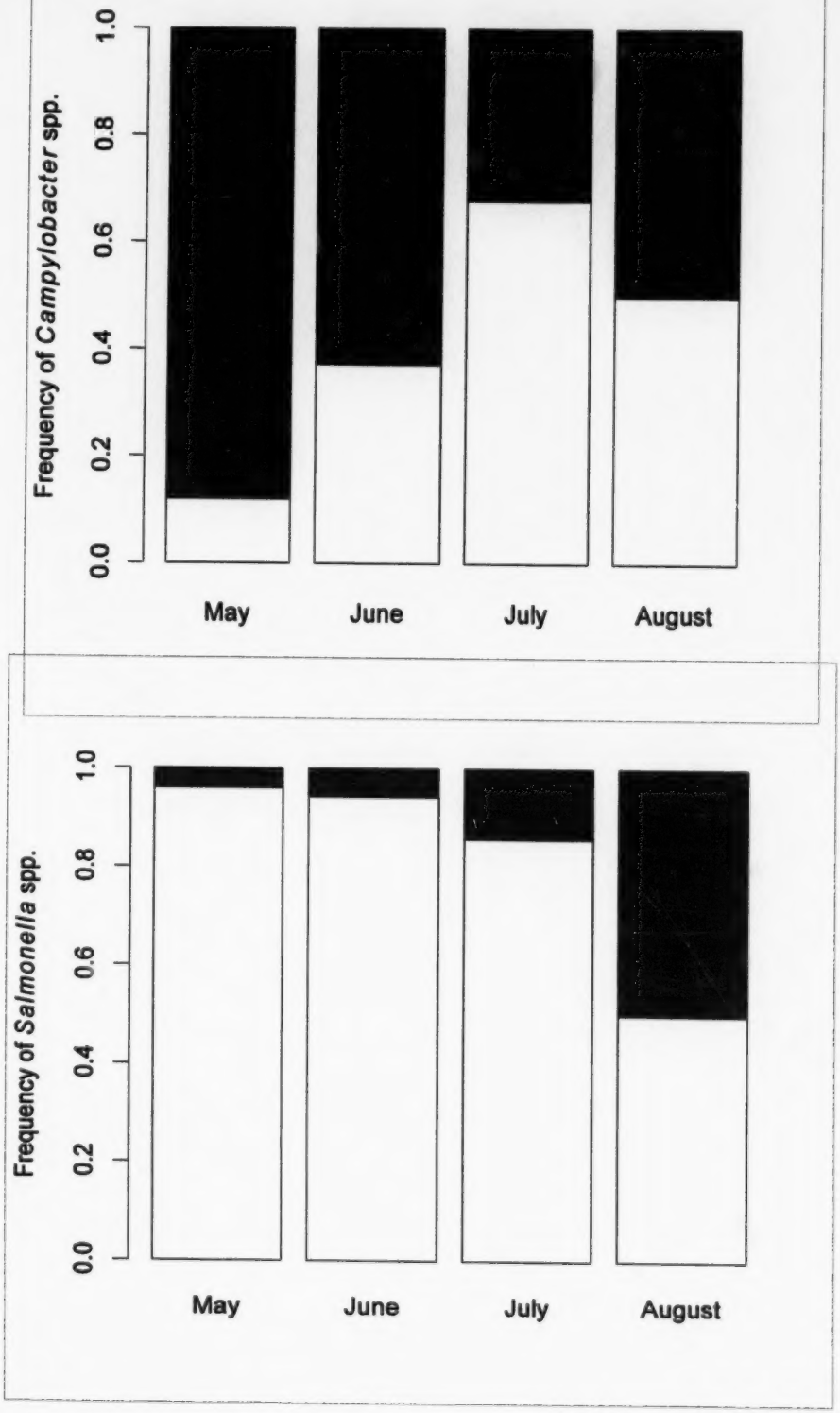


Figure 12. Relationship between the Bacteroidales markers and the pathogen *Campylobacter* during the 2009 sampling season.

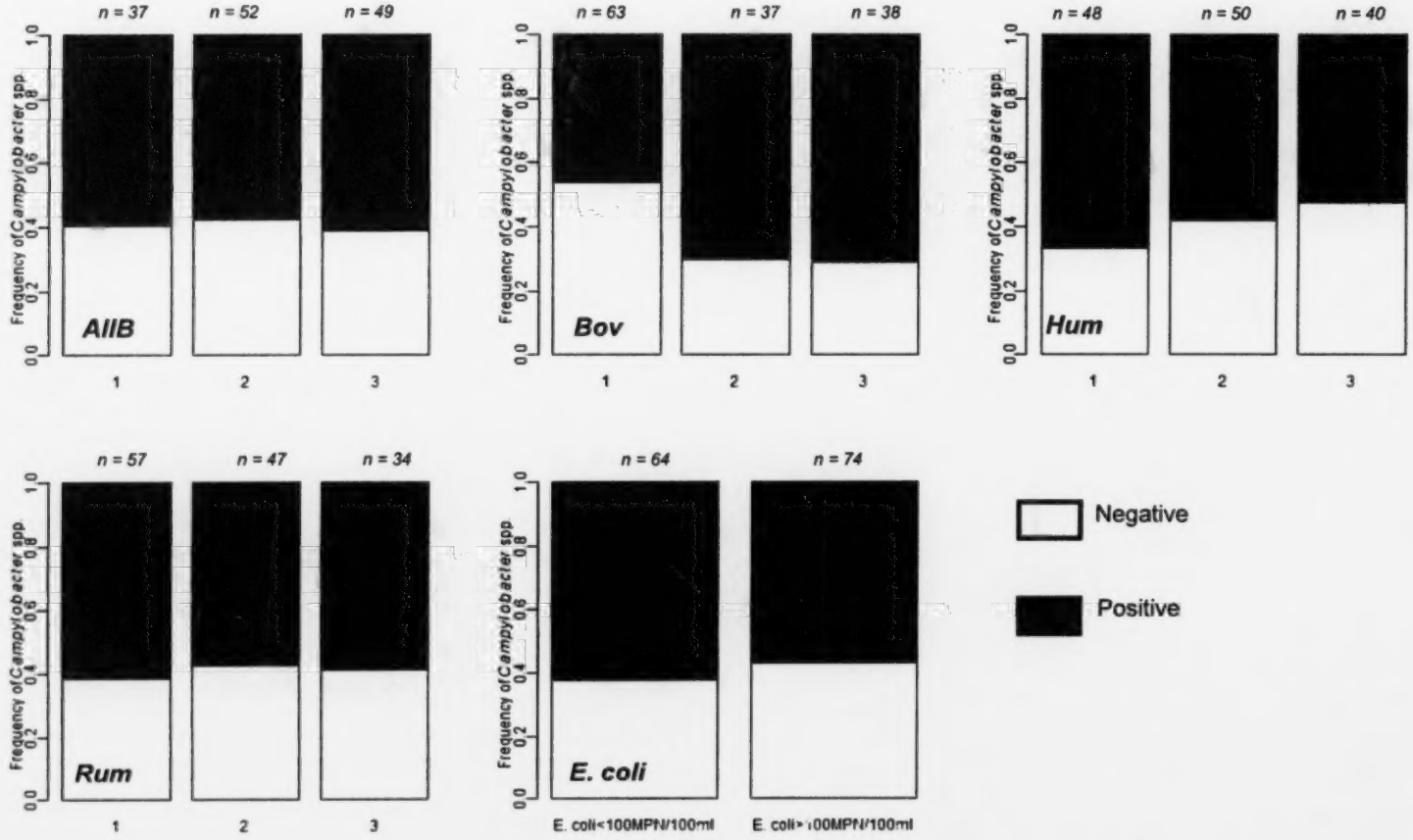


Figure 12. Relationship between the Bacteroidales markers and the pathogen *Campylobacter* during the 2009 sampling season.

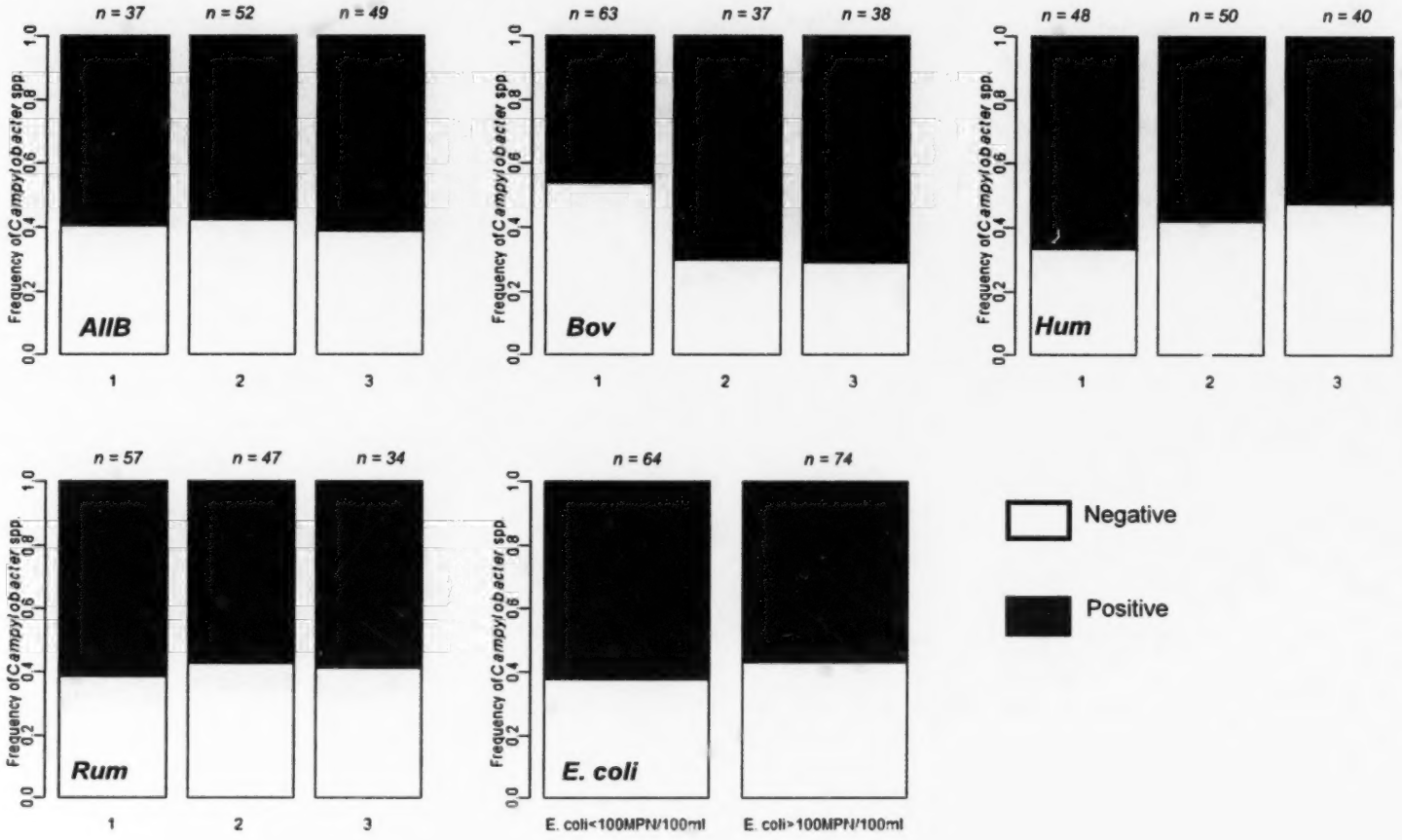
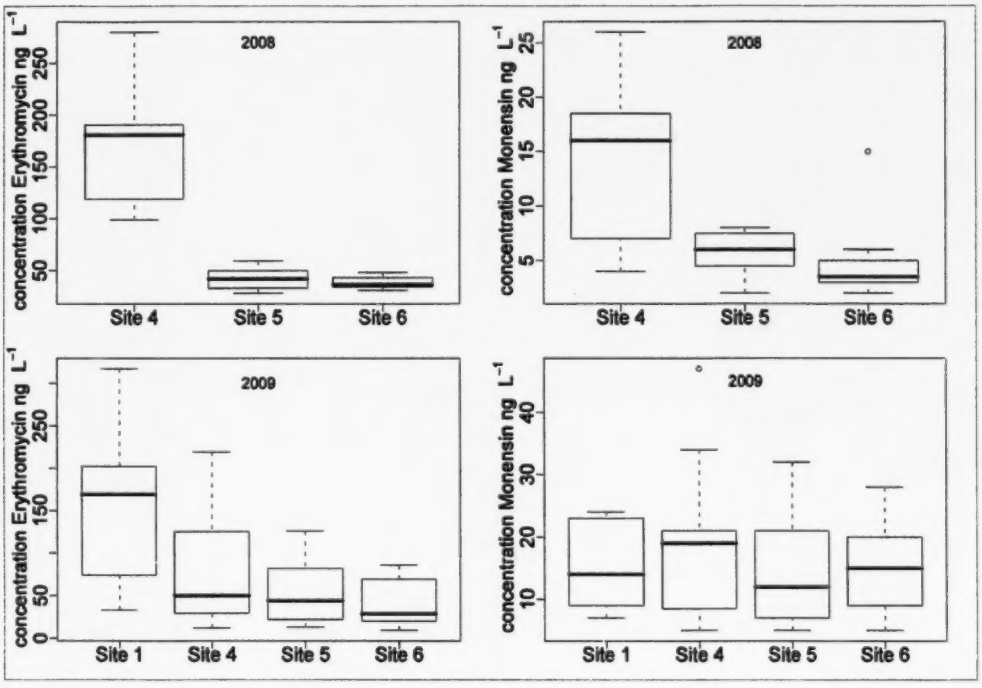
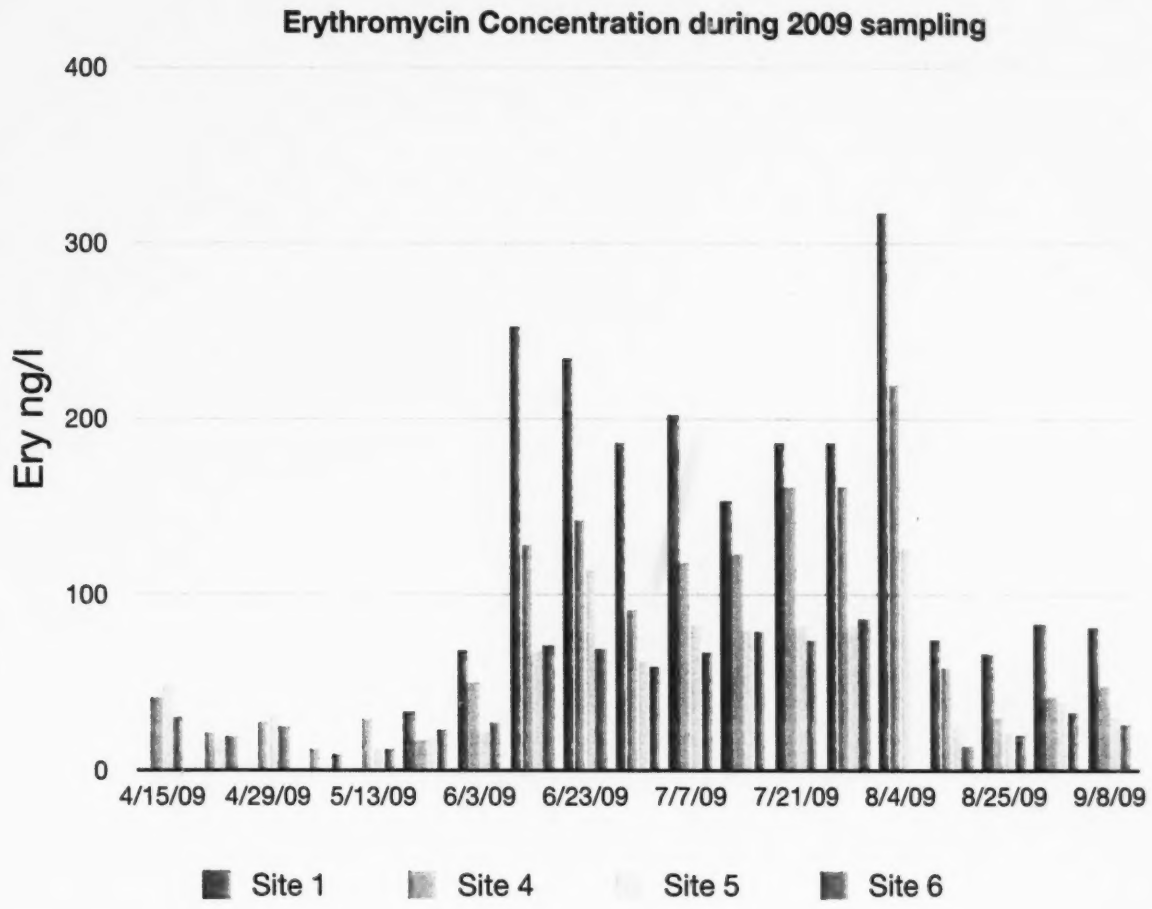


Figure 13. Detection of erythromycin and monensin in the Qu'Appelle River and Wascana Creek during the 2008 and 2009 sampling seasons.



Concentrations of monensin and erythromycin were significantly different between sampling sites in 2008 (Kruskal Wallis, $P < 0.01$). In 2009, there was a significant difference between sampling sites only when testing erythromycin concentrations (Kruskal Wallis, $P < 0.001$).

Figure 14. Weekly detection of eythromycin during the 2009 sampling season.



Note: Sampling at site 1 did not begin until late May.

Figure 15: Erythromycin concentrations at site 4 maybe influenced by concentrations at site 1.

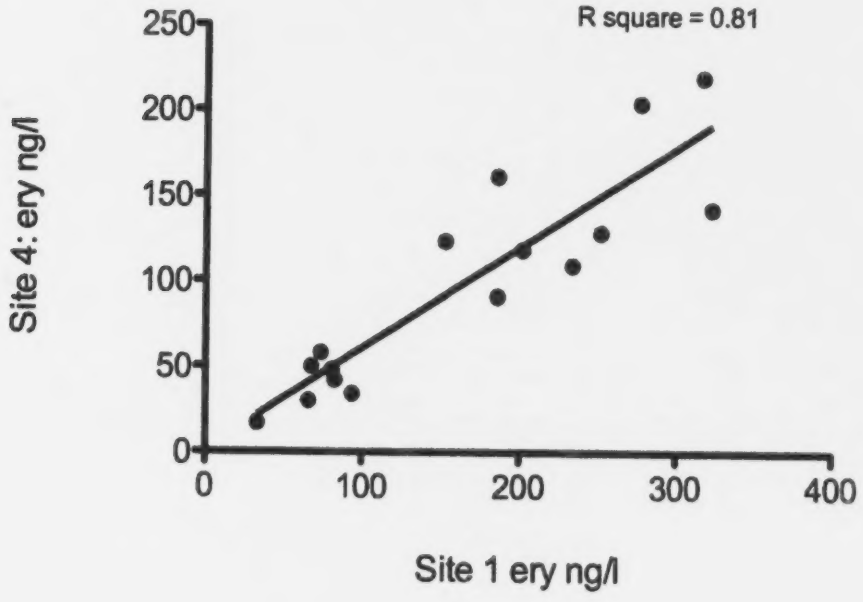
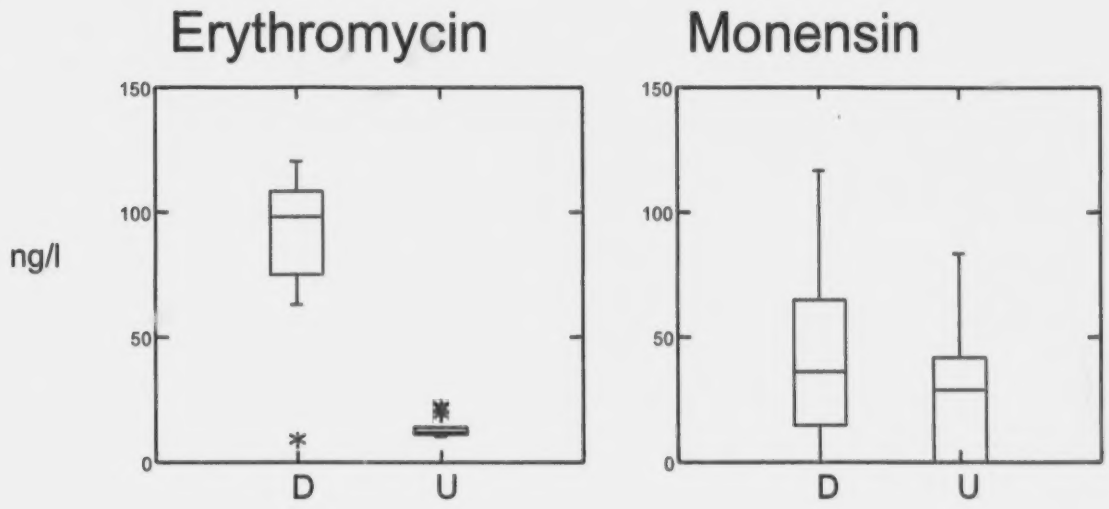


Figure 16. Erythromycin and monensin concentrations at sites on Wascana Creek upstream and downstream of the Regina wastewater treatment facility.



D, is the sampling site downstream of the treatment plant while U is the site upstream of the treatment plant. There was a significant difference between the sampling sites for erythromycin (Kruskal Wallis, $P < 0.001$) but not for monensin.

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Evaluation of host-specific *Bacteroidales* 16S rRNA gene markers as a complementary tool for detecting fecal pollution in a prairie watershed

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ABSTRACT

Our ability to identify and eliminate fecal contamination of water, now and in the future, is essential to reduce incidences of waterborne disease. Bacterial source tracking is a recently developed approach for identifying sources of fecal pollution. PCR primers designed by Bernhard and Field [Bernhard, A.E., Field, K.G., 2000a. A PCR assay to discriminate human and ruminant feces on the basis of host differences in *Bacteroides–Prevotella* genes encoding 16S rRNA. Appl. Environ. Microbiol. 66(10), 4571–4574] and Dick et al. [Dick, L.K., Bernhard, A.E., Brodeur, T.J., Santo Domingo, J.W., Simpson, J.M., Walters, S.P., Field, K.G., 2005. Host distributions of uncultivated fecal *Bacteroidales* bacteria reveal genetic markers for fecal source identification. Appl. Environ. Microbiol. 71(6), 3184–3191] for the detection of human (HF183), pig (PF163) and ruminant (CF128) specific *Bacteroidales* 16s rRNA genetic markers were tested for their suitability in detecting fecal pollution in Saskatchewan, Canada. The sensitivity and specificity of these primers were assessed by testing eight raw human sewage samples and 265 feces from 12 different species in Saskatchewan. The specificity of each primer set was ≥94%. The accuracy of HF183 and PF163 to distinguish between the different species was 100%, whereas CF128 cross-reacted with 22% of the pig feces. Occurrence of the host-specific *Bacteroidales* markers and the conventional indicator *Escherichia coli* in relation to several enteropathogens was investigated in 70 water samples collected from different sites along the Qu'Appelle River (Saskatchewan, Canada). Human and ruminant fecal markers were identified in 41 and 14% of the water samples, respectively, whereas the pig marker was never detected in the river water. The largest concentrations in *E. coli* counts were concomitant to the simultaneous detection of HF183 and CF128. Thermotolerant *Campylobacter* spp., *Salmonella* spp. and Shiga toxin genes (stx1 and stx2)-positive *E. coli* (STEC) were detected in 6, 7 and 63% of the water samples, respectively. However, none of the stx positive water samples were positive for the *E. coli* O157:H7 gene marker (*uidA*). Odds ratios analysis suggests that CF128 may be predictive for the presence of *Salmonella* spp. in the river investigated. None of the fecal indicators were able to confidently predict the presence of thermotolerant *Campylobacter* spp. and STEC.

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1. Introduction

Fecal pollution of freshwater can have lethal consequences when enteric pathogens are present in the fecal material (Hrudey et al., 2003; O'Reilly et al., 2007; Yoder et al., 2008). The public health risks from exposure to fecal dwelling pathogenic microorganisms are substantial, and in addition to the human tragedy, significant economic loss to a community can occur. Our ability to identify and eliminate fecal contamination, now and in the future, is essential to reduce incidences of waterborne disease. The low concentration and large diversity of enteric pathogens that can contaminate a watershed preclude the direct monitoring of water sources for presence of pathogens. Therefore, their potential presence in the aquatic environment relies mainly on the cultivation and enumeration of fecal indicator bacteria such as total and fecal coliforms and members of *Enterococcus* spp. (Savichtcheva and Okabe, 2006). However, some studies have shown that these conventional fecal indicators are poorly correlated with the presence of pathogenic microorganisms such as *Salmonella* or *Campylobacter* spp., and only indicate the overall pollution of a water body (Field and Samadpour, 2007). Moreover, detection of these indicators in water does not provide information regarding the possible sources of the fecal contamination. Identifying the source of the contamination in water can provide critical information necessary for development of rational and effective mitigation strategies, such as better treatment facilities for domestic and industrial effluents and/or management measures for agricultural sources.

Numerous microbial source tracking (MST) methods are being developed to help identify sources of fecal pollution in water. These methods can be divided into two groups: culture-based (e.g. antibiotic resistance profile or DNA fingerprinting) and culture-independent (e.g. chemical or DNA amplification methods) (Santo Domingo et al., 2007). Some of the culture-based strategies, such as pulse field gel electrophoresis (PFGE), ribotyping or multiple antibiotic resistance methods, have been conducted with *Escherichia coli* and enterococci. However, they are often labour-intensive requiring the development of a geographically defined host-origin database using significant numbers of isolates from fecal samples of known origin. In addition, some researchers question the host-specificity of these indicators and emphasize that they can proliferate and establish populations in natural environment, which may interfere in the tracking of recent contamination events (Field and Samadpour, 2007).

Certain species from the anaerobic *Bacteroidales* order have been suggested as alternative biological indicators of fecal pollution according to their host-specific distributions, their short survival rate once released into the natural environment (due to their low oxygen tolerance) and their abundance in feces from warm-blooded animals (Fiksdal et al., 1985; Savichtcheva and Okabe, 2006). The considerable advantage of these alternative indicators is their possible application in determining the source of fecal contamination by using a culture-independent molecular-based approach, which produces quick and meaningful results. Among them, PCR amplification of 16S rDNA from host-specific *Bacteroidales* has been described as a promising and rapid MST strategy. More precisely, host-specific *Bacteroidales* 16S rRNA genetic markers were recently identified and PCR primers have been designed for detection of human (HF183F–Bac708R), ruminant (CF128F–Bac708R) and swine (PF163F–Bac708R) specific feces (Bernhard and Field, 2000a; Dick et al., 2005). Several studies suggest that the host-specificity of the HF183 and PF163 markers may not be geographically constrained, whereas the specificity of CF128 is unresolved in several European countries (Ahmed et al., 2008; Gawler et al., 2007; Walters et al., 2007). To the best of our knowledge, detection of these markers has not been conducted in a Saskatchewan context, and this validation is a critical step before their use for source tracking of fecal contamination within Saskatchewan. Moreover, further studies are clearly required to validate an efficient combination of fecal contamination indicators for predicting the presence of pathogens in water and tracing their origin.

The main objective of this study was thus to evaluate the suitability of the host-specific *Bacteroidales* 16s rRNA PCR primers (HF183F, CF128F and PF163F paired with Bac708R) for identifying sources of fecal pollution in freshwater in Saskatchewan. The specificity and sensitivity of the PCR approach for detection of the human, pig and ruminant-specific *Bacteroidales* markers were evaluated by testing eight raw sewage samples and 265 feces originating from 12 different species in Saskatchewan. The PCR detection thresholds of the different primer sets were assessed and compared to the standard Colilert-18/Quanti-Tray technology for detecting *E. coli* in water. The presence of the host-specific *Bacteroidales* markers and the conventional indicator *E. coli* in relation to several enteropathogens (i.e. *Campylobacter coli*, *Campylobacter jejuni*, *Campylobacter lari*, *Salmonella* spp. and Shiga toxin genes (*stx1*, *stx2*)-positive *E. coli* (STEC) was evaluated by analysing freshwater taken from various sampling sites along the Qu'Appelle River (Saskatchewan, Canada).

2. Materials and methods

2.1. *Sampling design and sample collection*

2.1.1. *Fecal and raw sewage sample acquisitions*

To determine the specificity and sensitivity of the different *Bacteroidales* primer sets, a total of 265 individual fresh fecal samples were aseptically collected from 12 different host groups and eight human derived raw sewage samples, which were obtained from four localities in Saskatchewan (Prince Albert, Lloydminster, North Battleford and Regina) from November 2007 to August 2008. Individual human specimens ($n = 54$) were provided by the Disease Control Laboratory of Saskatchewan. Cow ($n = 51$), chicken ($n = 21$) and part of the pig feces ($n = 11$) were collected as certainly as possible from separate animals from southern SK farms. Additional fecal samples from pigs ($n = 39$) were obtained from the Prairie Swine Centre (Saskatoon, SK). Fecal samples from other animal species ($n = 80$) were taken from the Calgary zoo (Alberta), except the goose samples ($n = 20$) that were collected from the Wascana Lake (Regina, SK, Canada). Samples were transported to the laboratory on ice and kept at 4 °C until the time of analysis.

2.1.2. *Qu'Appelle River water samples*

Occurrence of the host-specific *Bacteroidales* markers and the conventional indicator *Escherichia coli* in relation to that of several enteropathogens was field evaluated in the Qu'Appelle River Basin of southern SK. The Qu'Appelle River extends over 400 km from headwaters near Lake Diefenbaker to join the Assiniboine River in Manitoba. More than 95% of the drainage area is composed of agricultural fields (wheat, barley, canola) and pastures (especially cattle), but the Qu'Appelle River also runs through urbanized areas such as the towns of Lumsden and Craven and is under the influence of effluent from the city of Regina sewage treatment facility (Hall et al., 1999).

Irrigation of crops in the Qu'Appelle Valley rely on source water from the Qu'Appelle River which was previously monitored for its microbial quality (based on enumeration of *E. coli*) in our laboratory (Fremaux et al., 2009). Similar to this earlier study, various sites were sampled weekly from June to September 2008; representing the times of most frequent irrigation. Sites included the Lumsden town (termed as Lumsden, ❶), two sites (termed as Wascana, ❷ and Qu'Appelle, ❸) both located upstream of Lumsden and two locations close to irrigation pumps used to irrigate vegetable crops. The first one is located immediately downstream of Lumsden (termed as IRPl, ❹) and the second downstream of Craven (termed as IRPc, ❺) (Fig. 1). Sampling at the Wascana site was used to determine the contribution of the Wascana creek to fecal contamination, one of the major tributaries of the Qu'Appelle River. At weekly intervals and for each site, three grab samples (at the middle and each edge of the river) of approximately 300 mL were taken from the surface water. The three samples were used separately for enumeration of *E. coli* using the Colilert-18/Quanti-Tray detection system (IDEXX Laboratories, Westbrook, Maine, UK) or pooled together for PCR detection of the different *Bacteroidales* markers and bacterial pathogens. In total, 70 river water samples (×3 for *E. coli* analysis) were collected, transported back to the laboratory on ice within 4 h of collection and subjected to microbiological analysis.

2.2. *Enumeration of E. coli by using the Colilert-18/Quanti-Tray system*

This method was used to process river water samples including those used for determining the detection threshold of the *Bacteroidales* PCR assays (cf. below). Each undiluted or appropriately diluted water sample (100 mL) was added to the Colilert-18 reagent and poured into a 97-well Quanti-Tray system, sealed and incubated at 37 °C for 22 h according to the manufacturer's instructions (IDEXX). Total coliforms and *E. coli* were simultaneously detected and enumerated by counting the number of yellow wells and yellow/fluorescent wells under UV light, respectively. The most probable number (MPN) could be estimated from a chart provided by the manufacturer. *E. coli* is a more specific indicator of fecal

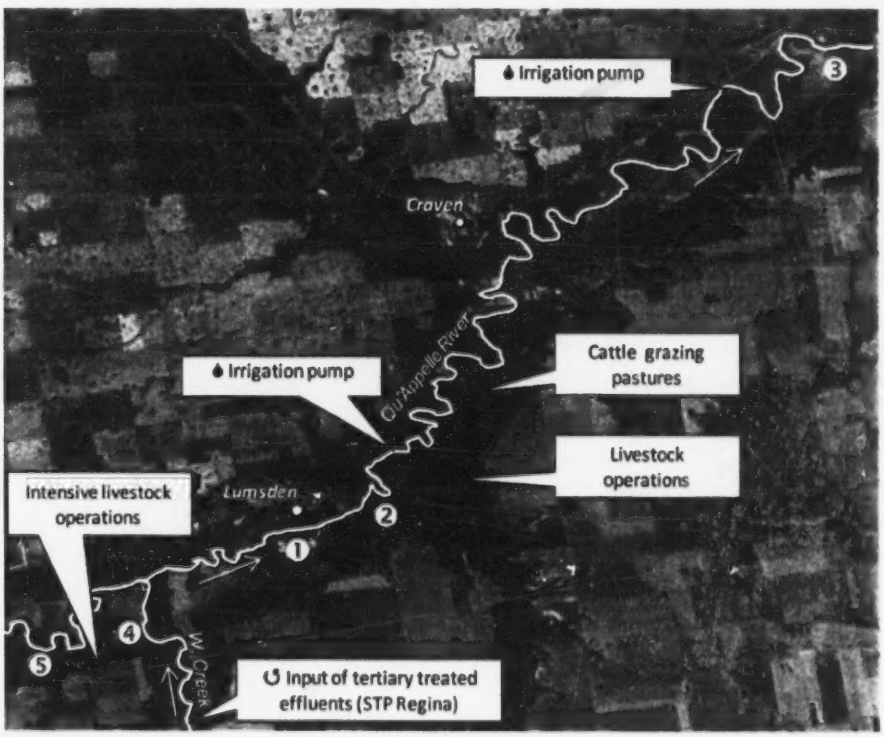

Fig. 1 – Aerial view of the different sampling sites (❶, Lumsden; ❷, IRPl; ❸, IRPc; ❹, Wascana; ❺, Qu'Appelle) along the Qu'Appelle River. The arrows indicate the direction of flow. Potential anthropogenic inputs are also indicated on the figure (Aerial photograph courtesy of http://www.Earth.Google.com).

contamination (Buckalew et al., 2006; Wilkes et al., 2009), therefore only data observed for these bacteria were shown in our study (results were reported as MPN per 100 mL).

2.3. *Sample preparation and DNA extraction for* Bacteroidales *and bacterial pathogen detection*

2.3.1. *Analysis of host-specific Bacteroidales*

DNA was directly extracted from 0.25 g of fecal material or 0.25 mL of raw sewage by using the Power Soil DNA kit (MoBio Laboratories Inc., Carlsbad, CA) according to the manufacturer's instructions. Water samples from the Qu'Appelle River were first concentrated by filtering 200 mL of water through a 0.45 μm pore size membrane using a *filter* funnel and vacuum system (Millipore Inc., USA). For each sample, the filter was placed in a sterile 15 mL falcon tube (Fisher Scientific, Ottawa, ON, Canada) containing 5 mL of sterile distilled water. The tubes were thoroughly vortexed and 2 mL of this solution was centrifuged at 11,000 × *g* for 3 min. The pellet was resuspended in 250 μl of sterile distilled water and DNA was extracted from this suspension by using the Power Soil DNA kit (MoBio Laboratories Inc.) according to the manufacturer's instructions.

2.3.2. *Detection of STEC, E. coli O157:H7, Salmonella spp. and Campylobacter spp*

The method of Bouvet et al. (2002) was employed for detection of STEC in water with slight modifications. Briefly, 200 mL of river water from each sampling site were filtered through a 0.45 μm pore size membrane (Millipore) and the filter was placed in a stomacher bag with 125 mL of buffered peptone water (BPW) (Sigma, Steinheim, Germany), manually mixed and incubated at 37 °C overnight. BPW without antibiotic has been recommended by Hussein and Bollinger (2008) when testing for stressed or injured STEC cells, as is the case in environmental samples. To increase the likelihood of detecting E. coli O157:H7, the most frequently implicated STEC causing human illness (Fey et al., 2000), a 1 mL aliquot of the BPW culture was transferred to 9 mL of modified trypticase soy broth (TSB) (Oxoid, Basingstoke, England) containing 20 μg/ml novobiocin (Sigma) and incubated at 42 °C for 6 ± 2 h (Johnson et al., 2003; Walters et al., 2007). Following enrichment the PCR assay described by Cebula et al. (1995) was used to detect the single base-pair mutation on *uidA* gene (position +92) of *E. coli* O157:H7. *Salmonella* spp. were selectively enriched by transferring 100 μl of the BPW cultures (above) to 10 mL of Rappaport–Vassiliadis soya peptone broth (RVB) (Oxoid) followed by incubation at 42 ± 1 °C for 24 h. *Campylobacter* spp. were cultured using a modified procedure of Walters et al. (2007). Accordingly, 200 mL of each water sample were filtered as described above and the membrane was incubated in 40 mL of Bolton enrichment broth (BB) (Oxoid) with selective supplement (Oxoid SR183E) plus 5% (v/v) laked horse blood (Oxoid). Incubation was performed under microaerophilic conditions at 42 °C for 48 h.

Following incubation and for each sample, 0.5 mL of each enrichment broth (BPW, TSB, RVB and BB) were mixed together (final volume 2 mL) and centrifuged at 11,000 × g for 5 min. The supernatant was gently removed and the pellet was resuspended in 250 μl of sterile distilled water. This suspension was used for DNA extraction by using the Power Soil DNA kit (MoBio Laboratories Inc.). Positive and negative control samples were included from the start of each extraction process, through amplification to electrophoresis. The positive control contained total DNA from the *E. coli* O157:H7 reference strain 43889 and from clinical isolates of *S. typhimurium* (ID 1399EN08), *C. lari* (ID 2039EN07), *C. jejuni* (ID 2065EN08) and *C. coli* (ID 1979EN08) provided by the Saskatchewan Disease Control Laboratory (Regina, SK, Canada).

2.4. PCR *analysis*

2.4.1. *Detection of host-specific Bacteroidales genetic markers*

PCR was used to detect host non-specific, human, ruminant and pig-specific *Bacteroidales* 16S rRNA gene markers in fecal (including raw sewage) and water samples using primer pairs previously designed by Bernhard and Field (2000a,b) (Table 1). All amplification reactions were processed in the MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA), and carried out in a total volume of 25 μl reaction mixture containing 5 μl of template DNA, 2 mmol l^{-1} $MgSO_4$, 0.2 mmol l^{-1} of each deoxynucleoside triphosphate (Invitrogen, Carlsbad, USA), 0.4 μmol l^{-1} of each primer (Sigma, Ontario, Canada), 1X Taq reaction buffer, 3 μg of Bovine Serum Albumine (Sigma, Ontario, Canada) and 1 U of Taq DNA polymerase (UBI, Calgary, Canada). The amplification program used was as follows: 5 min initial denaturation of DNA at 95 °C, followed by 30 cycles of 30 s denaturation at 94 °C, 30 s primer annealing at the temperature specific for each primer pair (Table 1), and 1 min extension at 72 °C. Amplification was completed by a final extension step at 72 °C for 6 min. PCR products were separated by gel electrophoresis on 1.5% agarose and visualized by ethidium bromide staining.

2.4.2. *Internal amplification control (IAC) for the Human-specific Bacteroidales PCR*

Although DNA eluates obtained by the Power Soil DNA kit (MoBio Laboratories) are of sufficient quality to be directly used for PCR, a synthetic DNA IAC was designed to confirm that false–negative results due to Taq inhibitors that could be present in the DNA extract did not occur. The IAC, with the sequence 5′-ATCATGAGTTCACATGTCCGGACAATAAGAAAG TCATGGTTATTGGCTGCCTTGAAGAGGAATTTGATGTAATCTT ATATGATGTTTTGGAGATGTCACGAAGAACTCCGATTG-3′ was constructed as previously described by Ufnar et al. (2007). It contained the same primer binding sequences as the target human-specific *Bacteroidales* marker DNA (HF183) and was included in each human-specific *Bacteroidales* PCR reaction. An IAC concentration of 10^{-9} μM was determined as the most appropriate since it does not compete with the target amplification. Based on the IAC detection from the HF183 PCR assay, PCR inhibition was not detected in any of the fecal, sewage or water DNA extracts analyzed in the present study.

2.4.3. *Detection of bacterial pathogens*

Individual PCR assays were used to detect the stx genes of STEC using degenerate primers that amplify a conserved sequence of both stx1 and stx2 genes as described by Read et al. (1992) as well as the *uidA* gene of *E. coli* O157:H7 (Cebula et al., 1995), *invA* gene of *Salmonella* spp. (Chiu and Ou, 1996)

Table 1 – Oligonucleotide PCR primers used in this study.

Primers	Primer sequences	Target microorganism (gene)	Annealing temp (°C)	Amplicon size (bp)	References
Pathogens					
INVA-1	ACAGTGCTCGTTTACGACCTGAAT	*Salmonella* spp. (*invA*)	56	244	Chiu and Ou (1996)
INVA-2	AGACGACTGGTACTGATCGATAAT				
ICJ-UP	CTTAGATTTATTTTTATCTTTAACT	*C. jejuni* (16S–23S rDNA ITS)	46	349	Khan and Edge (2007)
ICJ-DN	ACTAAATGATTTAGTCTCA				
ICL-UP	CTTACTTTAGGTTTTAAGACC	*C. lari* (16S–23S rDNA ITS)	46	279	Khan and Edge (2007)
ICL-DN	CAATAAAACCTTACTATCTC				
ICC-UP	GAAGTATCAATCTTAAAAAGATAA	*C. coli* (16S–23S rDNA ITS)	46	72	Khan and Edge (2007)
ICC-DN	AAATATATACTTGCTTTAGATT				
ES149	CGAAATYCYCTCTGTATYTGYC	STEC (*stx*)	49	323	Read et al. (1992)
ES151	GARCRAAATAATTTATATGT				
PT2	GCGAAAACTGTGGAATTGGG	*E. coli* O157:H7 (*uidA*)	60	252	Cebula et al. (1995)
PT3	TGATGCTCCATAACTTCCTG				
Bacteroides markers					
Bac32F	AACGCTAGCTACAGGCTT	General *Bacteroidales* (16S rRNA)	58	676	Bernhard and Field (2000b)
Bac708R	CAATCGGAGTTCTTCGTG				
CF128F	CCAACYTTCCCGWTACTC	Ruminant *Bacteroidales* species (16S rRNA)	53	580	Bernhard and Field (2000a)
HF183F	ATCATGAGTTCACATGTCCG	Human *Bacteroidales* species (16S rRNA)	59	525	Bernhard and Field (2000a)
PF163F	GCGGATTAATACCGTATGA	Pig *Bacteroidales* species (16S rRNA)	57.4	563	Dick et al. (2005)

and the species-specific genes encoding 16S–23S rDNA internal transcribed spacer region of thermotolerant *Campylobacter* species (i.e. *C. coli*, *C. lari* and *C. jejuni*) (Khan and Edge, 2007) in the river water samples. Since *C. coli*, *C. jejuni* and *C. lari* are most often implicated as the causative agent of campylobacteriosis (Khan and Edge, 2007), we choose to limit the detection to these species-specifically. The primer sequences and expected sizes of amplicons for each PCR assay are described in Table 1. PCR was performed in 25 μl reaction mixtures identical in constituents and volumes to those described above for the *Bacteroidales* PCR assays, with the exceptions of the primers adjusted to a final concentration of 1 μM for the detection of the *stx* and *uidA* genes. DNA extracts from both enriched (cf. 2–3–2) or non-enriched (cf. 2–3–1) water samples were separately used as DNA template as an approach to confirm that the targeted pathogenic genes were initially present in viable cells. PCR products were verified by gel electrophoresis on 1.5% agarose as described above.

2.5. Detection threshold of the different host-specific Bacteroidales PCR assays

The detection threshold (SLOD) was determined by using the method described by Ahmed et al. (2008), with slight changes. Three of the raw sewage, human, cow and pig fecal samples were separately suspended in freshwater taken from the Qu'Appelle River at a ratio of 1:10. Serial dilutions ranging from 10^{-1} to 10^{-9} were performed for each sample. Each dilution was subjected to DNA extraction followed by PCR analysis for detection of the general *Bacteroidales* marker Bac32 and the appropriate species-specific *Bacteroidales* marker as described above. The SLOD was determined as the lowest dilution of fecal sample, which gave an amplicon of the expected size. In addition, *E. coli* were quantified in each dilution by using the Colilert-18 system (IDEXX) to provide relevant information about the comparison between the *E. coli* level and detection by PCR of the host-specific *Bacteroidales* markers.

2.6. Confirmation of PCR amplicons by DNA sequencing

DNA sequencing was carried out to confirm the identity of the PCR fragments obtained for detection of *Salmonella* spp. ($n = 5$), *C. coli* ($n = 2$), *C. jejuni* ($n = 2$) and for randomly selected PCR-amplified sequences ($n = 10$) from the *stx* genes primer pair. In addition, human ($n = 1$) and ruminant ($n = 2$) specific *Bacteroidales* PCR products, obtained from three river water samples associated with high *E. coli* levels, were subjected to sequencing to validate the PCR detection. Briefly, DNA amplicons to be sequenced were purified from agarose gels using the Gel Extraction Qiaex II kit (Qiagen, Mississauga, ON, Canada). Purified PCR products generated using host-specific *Bacteroidales* and ITS-specific *C. coli* primers were cloned using the TOPO TA Cloning kit as indicated by the manufacturer (Invitrogen, Carlsbad, CA). Prior to DNA sequencing, recombinant plasmid DNA from a single clone per TOPO cloning reaction was extracted using a GenElute Plasmid Mini Prep kit (Sigma, St Louis, MO). PCR products from all other cases were directly sequenced after purification. Sequencing was performed by the University of Calgary Core DNA Sequencing Service (Calgary, Alberta, Canada). Sequence similarity searches were carried out using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST).

The DNA sequencing and BLAST analysis using the GenBank database confirmed that the PCR reactions resulted in appropriate amplification. Overall, sequence similarities were greater than 97% identical between the sequenced amplicon and the appropriate DNA sequences in the GenBank database. The PCR amplicons showing the presence of *Salmonella* ($n = 5$) exhibited at least 99% similarity with the

invA gene of *Salmonella enterica* subsp. *Enteric*. Sequences of the PCR-amplified 16S–23S rDNA ITS genes from the positive samples for *C. jejuni* ($n = 2$) and *C. coli* ($n = 2$) had a high degree of similarity with previously sequenced *C. jejuni* susbsp. *jejuni* (98%) and *C. coli* (97%) genes, respectively. The sequences of the *stx* genes showed up to 97% similarity with *stx2d* ($n = 7$ out of 10 PCR-amplified *stx* genes), 98% similarity with *stx2c* ($n = 2$) and 99% similarity with *stx1* ($n = 1$). Finally, the *Bacteroidales* clones exhibited a high sequence identity (≥97%) to their species of origin when subjected to BLAST analysis.

2.7. Nucleotide sequence accession numbers

The *Bacteroidales* 16s rDNA sequences have been submitted to GenBank under accession numbers FJ716126, FJ716127 and FJ716128.

2.8. Data analysis

The sensitivity and specificity of the PCR primer sets were calculated as: sensitivity = $a/(a + c)$ and specificity = $d/(b + d)$, where '*a*' is the true positive (positive fecal samples for the marker of its own species); '*b*' is the false positive (positive fecal samples for the marker of another species; '*c*' is the false negative (negative fecal samples for the marker of its own species); '*d*' is the true negative (negative fecal samples for the PCR marker of another species) (Ahmed et al., 2008; Gawler et al., 2007).

The detection of the general and host-specific *Bacteroidales* markers in water samples according to their origins (with five modalities: IRPc, IRPl, Lumsden, Wascana and Qu'Appelle sites) and their level of *E. coli* (with two modalities: *E. coli* <100 MPN 100 mL^{-1} or *E. coli* >100 MPN 100 mL^{-1} scored as 0 and 1, respectively) was analysed, and the statistical significance was tested using the Fisher's Exact test (due to the small sample size). The Canadian Council of Ministers of the Environment uses 100 MPN 100 mL^{-1} as an indicator for irrigation water guidelines.

As previously described by Walters et al. (2007), the odds ratio (with their confidence intervals) were calculated to assess the likelihood of contamination with thermotolerant *Campylobacter* spp., *Salmonella* spp. or STEC according to the level of *E. coli* (scored as 0 when it was <100 MPN 100 mL^{-1} and 1 when it was >100 MPN 100 mL^{-1}) and the presence of the host-specific *Bacteroidales* markers (CF128 and HF183) in freshwater samples taken from the Qu'Appelle River. These statistical calculations were performed using the R software version 2.6.2 (Ihaka and Gentleman, 1996).

3. Results

3.1. Host-associated specificity of the Bacteroidales PCR primers

The general *Bacteroidales* 16S rRNA marker (Bac32) was detected in all the fecal and raw sewage samples tested (Table 2). The detection ability of the PCR assay with the human-specific primer (HF183) was 100% for raw sewages versus 94% (51/54) for human feces. Similarly, a high detection ability of 98% (119/121) and 100% (50/50) was obtained for the ruminant (CF128) and pig (PF163)-specific primers, respectively. In contrast, the other human (HF134) and ruminant (CF193) specific-markers described by Bernhard and Field (2000a) were rarely detected among the human (32%, 14/44) and cow (16%, 5/32) fecal samples tested, respectively, and were not considered further in this study.

Table 2 – PCR detection of the host-specific *Bacteroidales* markers in different species present in Saskatchewan, Canada.

Fecal samples from different species	No. of samples	Positive *Bacteroidales* markers detection			
		HF183F	CF128F	PF163F	Bac32F
Humans					
Individual	54	51	0	0	54
Raw sewage	8	8	0	0	8
Total human samples	62	59	0	0	62
Animals					
Cow	51	0	49	0	51
Pig	50	0	11	50	50
Chicken	20	0	0	0	20
Goose	20	0	0	0	20
Moose	10	0	10	0	10
Deer, white tailed	10	0	10	0	10
Deer, mule	10	0	10	0	10
Deer, Fallow	10	0	10	0	10
Caribou	10	0	10	0	10
Bison	10	0	10	0	10
Goat	10	0	10	0	10
Total animal samples	211	0	130	50	211

The CF128 marker was unable to distinguish between domestic (cow) and wild (moose, deer, caribou, bison and wild goat) ruminants. The HF183 and PF163 primers both exhibited a specificity of 100% suggesting that no cross-amplification with fecal DNA from the other species was observed. By comparison, the specificity of the CF128 primer to differentiate ruminant from the non-ruminant species was 92.8% (141/152) as the CF128 marker was also detected in 22% (11/50) of the pig feces.

3.2. Detection threshold (SLOD) of the Bacteroidales PCR assays

The SLOD of the Bac32 marker was identical in all the various sources of fecal material (i.e. 1×10^{-7}) (Table 3), whereas it was two orders of magnitude lower in sewage samples (i.e. 1×10^{-5}). Except for the human feces, the SLOD of each species-specific *Bacteroidales* PCR assay was reproducible from one sample to another, and was usually one order of magnitude lower than that obtained for Bac32. In fact, the CF128 and PF163 markers were both detected up to dilution 1×10^{-6} g of each cow and pig fresh feces, respectively (Table 3). The HF183 marker was detected up to dilution 1×10^{-4} in all the raw sewage samples, but its SLOD varied from 1×10^{-5} to 1×10^{-7} depending on the human fecal sample tested. This lack of reproducibility, whereas the SLOD of the general *Bacteroidales*

Table 3 – Detection thresholds of the different *Bacteroidales* PCR assays and *E. coli* in raw sewages and fresh feces from diverse origins.

Origin	Sample No.	Detection threshold (g feces or mL sewage)		
		Species-specific *Bacteroidales* marker[a]	General *Bacteroidales* marker	*E. coli*
Human feces (HF183)	1	1×10^{-5}	1×10^{-7}	3×10^{-7}
	2	1×10^{-5}	1×10^{-7}	1.4×10^{-6}
	3	1×10^{-7}	1×10^{-7}	4.2×10^{-7}
Raw sewages (HF183)	1	1×10^{-4}	1×10^{-5}	1×10^{-5}
	2	1×10^{-4}	1×10^{-5}	2.3×10^{-5}
	3	1×10^{-4}	1×10^{-5}	5.4×10^{-4}
Cow feces (CF128)	1	1×10^{-6}	1×10^{-7}	1.3×10^{-4}
	2	1×10^{-6}	1×10^{-7}	1×10^{-4}
	3	1×10^{-6}	1×10^{-7}	6.3×10^{-4}
Pig feces (PF163)	1	1×10^{-6}	1×10^{-7}	1.3×10^{-6}
	2	1×10^{-6}	1×10^{-7}	8×10^{-6}
	3	1×10^{-6}	1×10^{-7}	6.2×10^{-6}

a Each *Bacteroidales* marker tested with fecal or raw sewage samples has been noted between parenthesis in the column 'origin'.

marker Bac32 was found similar in the three human feces, suggests that the prevalence of the *Bacteroidales* species carrying the HF183 marker may vary between individuals.

By comparison, detection of *E. coli* was up to 10-fold more sensitive than detection of the HF183 in raw sewage and up to 100-fold in individual human feces (Table 3). The PF163-specific PCR assay was found consistently as sensitive as *E. coli* in the pig fecal samples tested. Finally, the sensitivity of the CF128 marker was 100-fold higher than that of *E. coli*, which was detected at levels of ca. 10^4 CFU g^{-1} cow feces.

3.3. Fecal pollution indicators in the Qu'Appelle River

3.3.1. Enumeration of *E. coli* using the Colilert-18 system

The Qu'Appelle River exhibited both temporal and spatial variability in contamination with *E. coli*. The Wascana site was the most frequently impacted by high *E. coli* levels during the sampling period, followed by the Lumsden and IRPl site (Table 4). In comparison, the Qu'Appelle and IRPc sites experienced minimal contamination with *E. coli*. A high concentration of *E. coli* was detected on August 12th at the Wascana, Lumsden and IRPl sites and reached values above 1600 MPN 100 mL^{-1} (Table 4).

3.3.2. PCR detection of the *Bacteroidales* 16S rRNA genetic markers

The general *Bacteroidales* 16S rRNA marker was detected in all the water samples collected from the different sites. The host-origin of a portion of the *Bacteroidales* populations could be determined by using the species-specific *Bacteroidales* 16S rRNA markers in 55% (39/70) of the water samples analyzed. Whereas the pig-specific *Bacteroidales* 16S rRNA marker was not detected in any of the water samples, the HF183 and CF128 specific *Bacteroidales* 16S rRNA markers were detected in 41% (29/70) and 14% (10/70) of the water samples taken throughout the Qu'Appelle River. As shown in Table 4, the HF183 or CF128-positive water samples were widely dispersed among the

Table 4 – Levels of *E. coli*, and incidence of the *Bacteroidales* 16s rRNA markers and bacterial enteric pathogens in the different sites sampled weekly along the Qu'Appelle River during the studying period.

Sites	*E. coli*			*Bacteroidales* incidences			Enteropathogen incidences			
	Min–Max (MPN 100 ml^{-1})	Mean[a] (MPN 100 ml^{-1})	Samples > limit (*n*/*N*) (%)[b]	Bac32	HF183 (%)	CF128 (%)	*Salmonella* spp. (%)	*Campylobacter* spp. (%)	*E. coli* O157:H7	STEC (%)
IRPc	10–183	40	1/14 (7)	14/14	6/14 (43)	1/14 (7)	0/14	0/14	0/14	7/14 (50)
IRPl	25–2131[c]	255	5/14 (36)	14/14	5/14 (36)	3/14 (21)	2/14 (14)	3/14 (21)	0/14	9/14 (64)
Lumsden	26–2275[c]	270	5/14 (36)	14/14	5/14 (36)	2/14 (14)	1/14 (7)	0/14	0/14	8/14 (57)
Wascana	164–1673[c]	414	14/14 (100)	14/14	6/14 (43)	3/14 (21)	2/14 (14)	0/14	0/14	12/14 (86)
Qu'Appelle	16–62	38	0/14 (0)	14/14	7/14 (50)	1/14 (7)	0/14	1/14 (7)	0/14	8/14 (57)

a The mean total amount of *E. coli* is calculated from the addition of the values obtained from water samples analyzed during the study period (June–September).

b *n*, number of water samples with an *E. coli* level > 100 MPN 100 mL^{-1}; *N*, number of total water samples analyzed.

c The maximum *E. coli* concentrations occurred simultaneously at the Wascana, Lumsden and IRPl sites on August 12th.

sampling sites, and the Fisher's exact test analysis revealed no significant difference between all sites.

3.3.3. PCR detection for the presence of bacterial enteric pathogens

PCR amplicons could only be detected following enrichment of the water samples indicating that pathogen loads were very low and that the targeted pathogenic genes were most likely initially present in viable cells. *Salmonella* and thermotolerant *Campylobacter* spp. were detected in seven (10%) out of a total of 70 water samples collected from four different sampling sites (Table 4). Overall, five (7%) water samples were positive for *Salmonella* spp. Among the *Salmonella* spp positive samples, two sampled from the IRPl site, were also positive for either *C. jejuni* or *C. coli*. A total of four (6%) water samples were positive for thermotolerant *Campylobacter* spp., including two for *C. jejuni* (from the IRPl site) and two for *C. coli* (from the Qu'Appelle and IRPl sites). Notably, samples collected from the Wascana, Lumsden and IRPl sites on August 12th, the date of the observed spike in *E. coli* counts, were positive for either thermotolerant *Campylobacter* or *Salmonella* spp., or both. In contrast, *C. lari* and *E. coli* O157:H7 were not detected in this study. Prevalence of STEC in water samples was 63% (44/70). No observable difference in distribution of the STEC-positive water samples was obtained among the different sampling locations (Table 4).

3.4. Correlation between *E. coli* counts, detection of species-specific Bacteroidales markers and presence of bacterial enteric pathogens

Detection of the host-specific *Bacteroidales* markers was significantly different in water samples according to their level of *E. coli* ($P < 0.01$, Fisher's exact test) (Fig. 2). There was a higher prevalence of HF183 and CF128 in water samples with an *E. coli* level above 100 MPN 100 mL^{-1} than in the other ones [68% (17/25) versus 38% (17/45)]. Notably, 80% (8/10) of the CF128-positive samples were associated with an *E. coli* level above 100 MPN 100 mL^{-1}. Both HF183 and CF128 markers were detected at the Wascana, Lumsden or IRPl sites on August 12th when large spikes in *E. coli* numbers and the presence of bacterial pathogens also impacted the water.

Among the water samples that tested positive for thermotolerant *Campylobacter* and/or *Salmonella* spp., six out of seven (86%) also contained an *E. coli* level above the 100 MPN 100 ml^{-1} limit and/or either the CF128 or HF183 marker, or both. This percentage dropped to 57% (25/44) when considering STEC-positive water samples.

Correlation between fecal indicators and the occurrence of bacterial pathogens was assessed by calculating the odds ratios and their 95% confidence intervals (Walters et al., 2007). It was not possible to define odds ratio for *Salmonella* spp., thermotolerant *Campylobacter* spp. and STEC relative to the

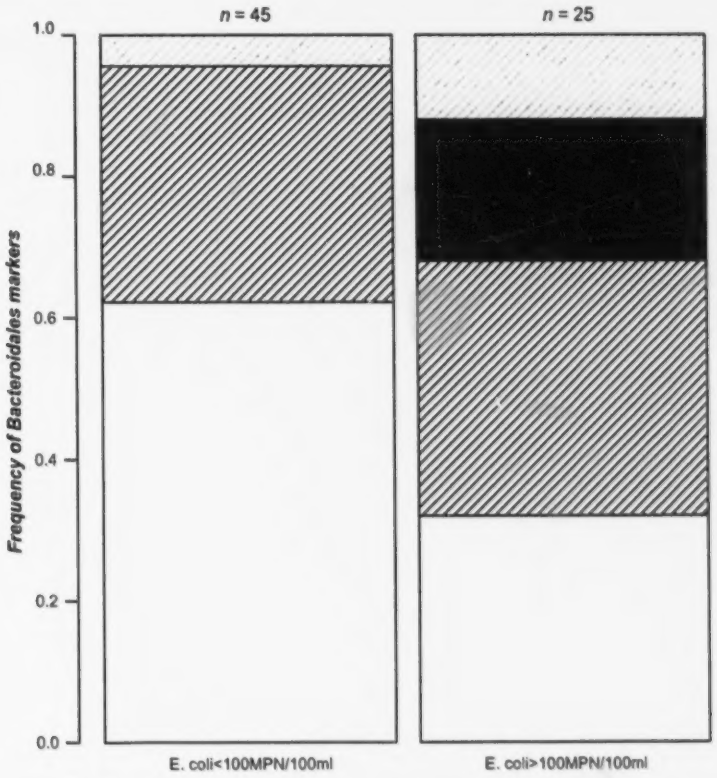

Fig. 2 – Detection of *Bacteroidales* markers (*white*, unknown origin; *shaded black*, human origin; *black*, ruminant origin; *shaded grey*, both human and ruminant origins) in water samples according to their level of *E. coli*.

general *Bacteroidales* marker Bac32 since this marker was present in all pathogen-positive samples.

There was no significant increased probability of detecting any of the bacterial pathogens in samples containing either an *E. coli* level above 100 MPN 100 mL^{-1} or the human *Bacteroidales* marker HF183, since the confidence interval of the odds ratios included values ≤1. Similarly, detection of CF128 showed no significant association with the occurrence of thermotolerant *Campylobacter* spp. or STEC in water. In contrast, presence of the CF128 marker was significantly correlated with the presence of *Salmonella* spp. in water. The odds of detecting *Salmonella* spp. when this marker was present were 12 times greater than when it was absent (OR 12.2; 95% CI from 1.7 to 86.2).

4. Discussion

The first aim of this study was evaluating the host-specificity of selected *Bacteroidales* 16S rRNA genetic markers using a large range of fecal samples (including raw sewages) originating from different species in Saskatchewan. The high sensitivities obtained for the HF183 (100% in raw sewages and 94% in individual human feces), CF128 (98%) and PF163 (100%) markers, indicate that the *Bacteroidales* host-specificity detected by this PCR method is present in Saskatchewan. This finding was comparable to those obtained for both the HF183 and CF128 markers in regions of Europe (Gawler et al., 2007) or the United States (Oregon) (Bernhard and Field, 2000a), and recently in Australia for HF183 (Ahmed et al., 2008). Similar to our study, the PF163 marker exhibited a 100% sensitivity when tested in France (Gourmelon et al., 2007). The *Bacteroidales* species that carry the HF183 or PF163 markers were strictly host-specific in our study as no cross-amplification with fecal DNA from other species was obtained. Conversely, fecal DNA extracted from 11 pig feces cross-reacted with the CF128 specific primer. The fact that this primer failed to differentiate between ruminant and pig (a non-ruminant) feces was previously reported in Europe (Gawler et al., 2007; Gourmelon et al., 2007). Therefore, detection of the CF128 marker alone indicates possible contamination with ruminant and/or pig feces. But as appropriately noted by Gourmelon et al. (2007), fecal pollution by pigs can be confirmed by detection of both CF128 and PF163 markers. In addition, the CF128 marker was unable to distinguish between domestic and wild ruminants, and consequently its presence in water does not necessarily indicate fecal contamination from agricultural origin.

The PCR assays allow sensitive detection of human, ruminant or pig fecal pollution. The results demonstrated that 1 g of pig or cow feces could still be detected after dilution in one cubic meter of water using the PF163 and CF128 specific primer, respectively. Notably, detection of the CF128 marker was found 100-fold more sensitive than detection of *E. coli* in the cow feces samples tested. Based on the same protocol, Bernhard and Field (2000a) reported similar CF128-SLOD values in cow feces (i.e. 10^{-6}–10^{-7} g feces). Using raw sewages, we demonstrated that the HF183 marker was consistently detected up to dilution 1×10^{-4} mL sewage, which is up to 10-fold less sensitive than *E. coli*. Two previous studies also reported the lower sensitivity of HF183 for detecting sewage than fecal coliforms (Bernhard and Field, 2000a; Bower et al., 2005). A possible explanation would be the uneven distribution and/or degradation of target DNA following the death of most bacteria in the *Bacteroidales* group due to their lower oxygen tolerance compared to fecal coliforms once released into sewage (Avelar et al., 1998; Savichtcheva and Okabe, 2006).

The host-specific *Bacteroidales* PCR assays were then field tested in the Qu'Appelle River (SK, Canada), a water source used routinely for crop irrigation and influenced by several different anthropogenic activities (Fig. 1). Prior monitoring of its microbial water quality revealed that levels of *E. coli* in the river frequently exceed current guidelines for water quality irrigation sources (100 fecal coliform CFU 100 ml^{-1}, Environment Canada, Canadian Water Quality Guidelines) (Fremaux et al., 2009). Therefore, identification of the sources of fecal pollution may represent a substantial advance in the restoration and protection of irrigation water quality through the implementation of appropriate mitigation strategies. Objectives of this part of the study were to evaluate the suitability of the *Bacteroidales* markers to detect fecal contamination in the Qu'Appelle River, and their occurrence relative to that of several enteropathogens.

All water samples tested positive for the general Bac32 *Bacteroidales* marker, suggesting that all sites experience some form of fecal pollution. The inability of *Bacteroidales* to survive under aerobic conditions, especially at high temperatures encountered during summer (Avelar et al., 1998; Kreader, 1998; Seurinck et al., 2005), combined with the recurrent detection of the Bac32 marker suggests that all sampling sites were regularly subjected to contamination by fecal material. However, further research is required to determine the persistence of this marker in environmental locations such as river sediments. In addition, the fact that indigenous uncultured environmental *Bacteroidales* populations could carry this marker should be considered (Lee et al., 2008). Quantification of the conventional indicator *E. coli* suggests that the degree of exposure to the sources of fecal pollution strongly differed among the sampling locations. The detection of *E. coli* levels above the recommended limit of 100 CFU per 100 mL (Jones and Shortt, 2005) ranged from 100% in water samples collected from the Wascana site to 0% in those from the Qu'Appelle site.

The use of host-specific *Bacteroidales* gene markers revealed that the Qu'Appelle River was occasionally impacted by human and ruminant fecal contamination as the HF183 and CF128 markers were detected in 41 and 14% of the water samples analysed, respectively, without significant difference between the sampling locations. In contrast, the pig-specific PF163 marker was never detected at any of the sampling sites. This is not surprising given the low amount of pig farming activity reported in the Qu'Appelle Valley.

The HF183 and CF128 markers were preferentially detected in water samples associated with a level of *E. coli* above 100 MPN 100 ml^{-1}. Notably, 80% of the CF128-positive water samples contained a concentration in *E. coli* above 100 MPN 100 ml^{-1}. The unusually high *E. coli* concentrations (above 1600 MPN 100 mL^{-1}) measured on August 12th at the Wascana, Lumsden and IRPl sites, were associated with the simultaneously detection of both the HF183 and CF128 markers. However, the relative contribution of each of these markers to the spikes in *E. coli* counts could not be assessed

using the qualitative PCR assays. Real-time quantitative PCR methods have been recently developed by several different international groups and used to quantify bovine and human-specific *Bacteroidales* markers in water samples (Layton et al., 2006; Kildare et al., 2007; Okabe et al., 2007; Reischer et al., 2007; Shanks et al., 2008), which can help to determine the various contributions of fecal contamination in cases of mixed sources. Notably, the Wascana creek continuously receives discharges of effluents from the sewage treatment plant (Regina) that may affect downstream water quality in case of system failure. In addition, the Qu'Appelle River is surrounded by numerous livestock operations (Fig. 1), and inappropriate agricultural practices such as direct deposition of cattle waste into water could lead to both large *E. coli* spikes and detection of the ruminant-specific marker. The results of this study emphasize the need for a comprehensive watershed-scale assessment of fecal pollution in the Qu'Appelle Valley watershed that should include hydrological studies, and multiple seasons of data collection. The *Bacteroidales* gene markers investigated in this study may provide a valuable tool to help define the fecal inputs in such a comprehensive study.

Salmonella spp., thermotolerant *Campylobacter* spp. and STEC non-O157:H7 were detected in the Qu'Appelle river water during the irrigation season. STEC were detected at high frequency regardless of the sampling sites and were frequently found in water relatively unpolluted as indicated by the absence of either conventional or alternative fecal indicators. However, it is important to note that not all STEC strains carrying the *stx* genes may be pathogenic to humans. The virulence of STEC is multifactorial, and further evaluation of their serotypes and detection of other additional virulence genes, for example intimin (*eae*) and enterohemolysin (*ehxA*) are required to assess the virulence potential of the STEC strains (Nataro and Kaper, 1998). Because ruminants, especially cattle, are considered as the main reservoir of STEC, it was expected to find a correlation with CF128. The lack of correlation between this marker and STEC may be caused by the ability of STEC to persist in freshwater (Fremaux et al., 2008; Watterworth et al., 2006) for a period of time much longer than the CF128 specific *Bacteroidales* marker. This could be also due to the high dilution of the cow *Bacteroidales* marker in water which causes problems for their detection. In contrast, *Salmonella* and thermotolerant *Campylobacter* spp. were detected on few occasions, however detection did occur at the IRPl site, which is close to the irrigation pump used for irrigation of vegetable crops.

The odds ratios analysis revealed that the CF128 marker may predict the presence of *Salmonella* spp. in the river. This is consistent with the fact that *Salmonella* spp. are found naturally in the intestines of ruminant such as cattle (Khaitsa et al., 2007; Milnes et al., 2008; Pangloli et al., 2008), which are present in relatively high density in the watershed (Fig. 1). In contrast, none of the *Bacteroidales* host-specific fecal markers tested in this study as well as the conventional indicator *E. coli* were adequate to predict the presence of thermotolerant *Campylobacter* spp. However, due to the low detection rates of these pathogens (seven out of the 70 water samples) during the season, further data is required to confidently assess the relationship of *Salmonella* and thermotolerant *Campylobacter* spp. with such fecal indicators in this study system. Walters et al. (2007) found a significant association between HF183 and *Campylobacter* spp., but neither *E. coli* or the *Bacteroidales* markers were able to predict the presence of *Salmonella* spp. in surface water samples (Oldman River Basin, southern Alberta, Canada). Based on quantitative data obtained from WWTP and water surface samples, Savichtcheva et al. (2007) showed that fecal coliforms and the HF183 marker had the greatest predictive value for the occurrence of *Salmonella* spp. Discrepancies between these studies results from the fact that thermotolerant *Campylobacter* and *Salmonella* spp. may be carried by several host-organisms. As a consequence, the strength in the relationship between fecal source tracking indicators and presence of these pathogens may vary significantly between different watersheds according to the abundance and frequency of humans and/or animals carrying the pathogens.

5. Conclusions

- PCR detection of the various host-specific *Bacteroidales* markers in fecal samples exhibited a great host-specificity and sensitivity. Therefore, it can be a valuable additional tool available to researchers undertaking comprehensive studies to characterize fecal pollution inputs in the watershed.
- A statistically significant relationship was demonstrated between occurrence of the ruminant-specific *Bacteroidales* marker (CF128) and that of *Salmonella* spp.
- Their detection in the Qu'Appelle River emphasizes the benefit of combined measurement of both alternative and conventional indicators for a better understanding of the nature of fecal contamination events.

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Quantitative Real-Time PCR Assays for Sensitive Detection of Canada Goose-Specific Fecal Pollution in Water Sources▽†

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Canada geese (*Branta canadensis*) are prevalent in North America and may contribute to fecal pollution of water systems where they congregate. This work provides two novel real-time PCR assays (CGOF1-*Bac* and CGOF2-*Bac*) allowing for the specific and sensitive detection of *Bacteroides* 16S rRNA gene markers present within Canada goose feces.

The Canada goose (*Branta canadensis*) is a prevalent waterfowl species in North America. The population density of Canada geese has doubled during the past 15 years, and the population was estimated to be close to 3 million in 2007 (4). Canada geese often congregate within urban settings, likely due to available water sources, predator-free grasslands, and readily available food supplied by humans (6). They are suspected to contribute to pollution of aquatic environments due to the large amounts of fecal matter that can be transported into the water. This can create a public health threat if the fecal droppings contain pathogenic microorganisms (6, 7, 9, 10, 12, 13, 19). Therefore, tracking transient fecal pollution of water due to fecal inputs from waterfowl, such as Canada geese, is of importance for protecting public health.

PCR detection of host-specific 16S rRNA gene sequences from *Bacteroidales* of fecal origin has been described as a promising microbial source-tracking (MST) approach due to its rapidity and high specificity (2, 3). Recently, Lu et al. (15) characterized the fecal microbial community from Canada geese by constructing a 16S rRNA gene sequence database using primers designed to amplify all bacterial 16S rRNA gene sequences. The authors reported that the majority of the 16S rRNA gene sequences obtained were related to *Clostridia* or *Bacilli* and to a lesser degree *Bacteroidetes*, which represent possible targets for host-specific source-tracking assays.

The main objective of this study was to identify novel *Bacteroidales* 16S rRNA gene sequences that are specific to Canada goose feces and design primers and TaqMan fluorescent probes for sensitive and specific quantification of Canada goose fecal contamination in water sources.

Primers 32F and 708R from Bernhard and Field (2) were used to construct a *Bacteroidales*-specific 16S rRNA gene clone library from Canada goose fecal samples (n = 15) collected from grass lawns surrounding Wascana Lake (Regina, SK, Canada) in May 2009 (for a detailed protocol, see File S1 in the supplemental material). Two hundred eighty-eight clones were randomly selected and subjected to DNA sequencing (at the Plant Biotechnology Institute DNA Technologies Unit, Saskatoon, SK, Canada). Representative sequences of each operational taxonomic unit (OTU) were recovered using an approach similar to that described by Mieszkin et al. (16). Sequences that were less than 93% similar to 16S rRNA gene sequences from nontarget host species in GenBank were used in multiple alignments to identify regions of DNA sequence that were putatively goose specific. Subsequently, two TaqMan fluorescent probe sets (targeting markers designated CGOF1-*Bac* and CGOF2-*Bac*) were designed using the RealTimeDesign software provided by Biosearch Technologies (http://www.biosearchtech.com/). The newly designed primer and probe set for the CGOF1-*Bac* assay included CG1F (5′-GTAGGCCGTGTTTTAAGTCAGC-3′) and CG1R (5′-AGTTCCGCCTGCCTTGTCTA-3′) and a TaqMan probe (5′-6-carboxyfluorescein [FAM]-CCGTGCCGTTATACTGAG ACACTTGAG-Black Hole Quencher 1 [BHQ-1]-3′), and the CGOF2-*Bac* assay had primers CG2F (5′-ACTCAGGGATAG CCTTTCGA-3′) and CG2R (5′-ACCGATGAATCTTTCTTTG TCTCC-3′) and a TaqMan probe (5′-FAM-AATACCTGATGC CTTTGTTTCCCTGCA-BHQ-1-3′). Oligonucleotide specificities for the Canada goose-associated *Bacteroides* 16S rRNA primers were verified through *in silico* analysis using BLASTN (1) and the probe match program of the Ribosomal Database Project (release 10) (5). Host specificity was further confirmed using DNA extracts from 6 raw human sewage samples from various geographical locations in Saskatchewan and 386 fecal samples originating from 17 different animal species in Saskatchewan, including samples from Canada geese (n = 101) (Table 1). An existing nested PCR assay for detecting Canada goose feces (15) (targeting genetic marker CG-*Prev* f5) (see Table S1 in the supplemental material) was also tested for specificity using the individual fecal and raw sewage samples (Table 1). All fecal DNA extracts were obtained from 0.25 g of fecal material by using the PowerSoil DNA extraction kit (Mo Bio Inc., Carlsbad, CA) (File S1 in the supplemental material provides details on the sample collection).

The majority of the Canada goose feces analyzed in this study (94%; 95 of 101) carried the *Bacteroidales* order-specific genetic marker designated All-*Bac*, with a relatively high median concentration of 8.2 $\log_{10}$ copies g^{-1} wet feces (Table 1

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TABLE 1. Specificities of the CGOF1-*Bac*, CGOF2-*Bac*, and CG-*Prev* f5 PCR assays for different species present in Saskatchewan, Canada

Host group or sample type	No. of samples	No. positive for *Bacteroidales* marker:			
		CGOF1-*Bac*	CGOF2-*Bac*	CG-*Prev* f5	All-*Bac*
Individual human feces	25	0	0	1	25
Raw human sewage	6	0	0	0	6
Cows	41	0	0	0	41
Pigs	48	0	0	1	48
Chickens	34	0	0	8	34
Geese	101	58	51	59	95[a]
Gulls	16	0	0	6	14
Pigeons	25	1	0	2	22
Ducks	10	0	0	0	10
Swans	1	0	0	0	1
Moose	10	0	0	0	10
Deer					
White tailed	10	0	0	0	10
Mule	10	0	0	0	10
Fallow	10	0	0	0	10
Caribou	10	0	0	0	10
Bison	10	0	0	0	10
Goats	10	0	0	0	10
Horses	15	0	0	0	15
Total	392	59	51	77	381

[a] The 6 goose samples that tested negative for the All-*Bac* marker also tested negative for the three goose markers.

and Fig. 1). The high prevalence and abundance of *Bacteroidales* in Canada goose feces suggested that detecting members of this order could be useful in identifying fecal contamination associated with Canada goose populations.

The composition of the *Bacteroidales* community in Canada goose feces ($n = 15$) was found to be relatively diverse since 52 OTUs (with a cutoff of 98% similarity) were identified among 211 nonchimeric 16S rRNA gene sequences. Phylogenetic analysis of the 52 OTUs (labeled CGOF1 to CGOF52) revealed that 43 (representing 84% of the 16S rRNA gene sequences) were *Bacteroides* like and that 9 (representing 16% of the 16S rRNA gene sequences) were likely to be members of the *Prevotella*-specific cluster (see Fig. S2 in the supplemental material). Similarly, Jeter et al. (11) reported that 75.7% of the *Bacteroidales* 16S rRNA clone library sequences generated from goose fecal samples were *Bacteroides* like. The majority of the *Bacteroides*- and *Prevotella*-like OTUs were dispersed among a wide range of previously characterized sequences from various hosts and did not occur in distinct clusters suitable for the design of Canada goose-associated real-time quantitative PCR (qPCR) assays (see Fig. S2 in the supplemental material). However, two single *Bacteroides*-like OTU sequences (CGOF1 and CGOF2) contained putative goose-specific DNA regions that were identified by *in silico* analysis (using BLASTN, the probe match program of the Ribosomal Database Project, and multiple alignment). The primers and probe for the CGOF1-*Bac* and CGOF2-*Bac* assays were designed with no mismatches to the clones CGOF1 and CGOF2, respectively.

The CGOF2-*Bac* assay demonstrated no cross-amplification with fecal DNA from other host groups, while cross-amplification for the CGOF1-*Bac* assay was limited to one pigeon fecal sample (1 of 25, i.e., 4% of the samples) (Table 1). Since the abundance in the pigeon sample was low (3.3 $\log_{10}$ marker copies g^{-1} feces) and detection occurred late in the qPCR (with a threshold cycle [C_T] value of 37.1), it is unlikely that this false amplification would negatively impact the use of the assay as a tool for detection of Canada goose-specific fecal pollution in environmental samples. In comparison, the nested PCR CG-*Prev* f5 assay described by Lu and colleagues (15) demonstrated non-host-specific DNA amplification with fecal DNA samples from several animals, including samples from humans, pigeons, gulls, and agriculturally relevant pigs and chickens (Table 1).

Both CGOF1-*Bac* and CGOF2-*Bac* assays showed limits of quantification (less than 10 copies of target DNA per reaction) similar to those of other host-specific *Bacteroidales* real-time qPCR assays (14, 16, 18). The sensitivities of the CGOF1-*Bac* and CGOF2-*Bac* assays were 57% (with 58 of 101 samples testing positive) and 50% (with 51 of 101 samples testing positive) for Canada goose feces, respectively (Table 1). A similar sensitivity of 58% (with 59 of 101 samples testing positive) was obtained using the CG-*Prev* f5 PCR assay. The combined use of the three assays increased the detection level to 72% (73 of 101) (Fig. 2). Importantly, all markers were detected within groups of Canada goose feces collected each month from May to September, indicating relative temporal stability of the markers. The CG-*Prev* f5 PCR assay is an end point assay, and therefore the abundance of the gene marker in Canada goose fecal samples could not be determined. However, development of the CGOF1-*Bac* and CGOF2-*Bac* qPCR approach allowed for the quantification of the host-specific CGOF1-*Bac* and CGOF2-*Bac* markers. In the feces of some individual Canada

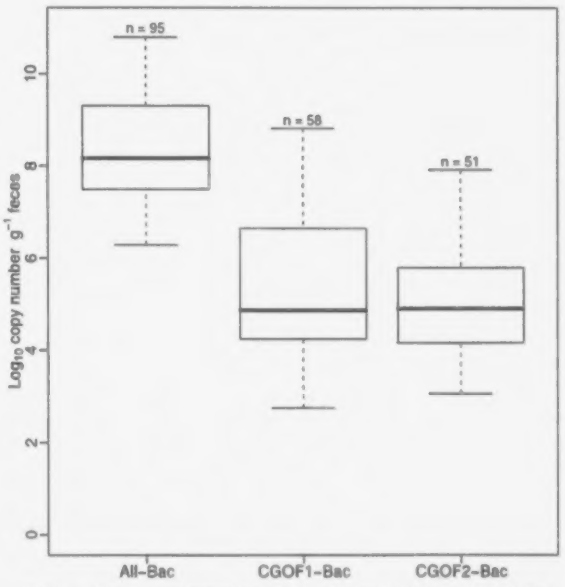


FIG. 1. Concentrations of the *Bacteroidales* (All-*Bac*, CGOF1-*Bac*, and CGOF2-*Bac*) genetic markers in feces from various individual Canada geese.

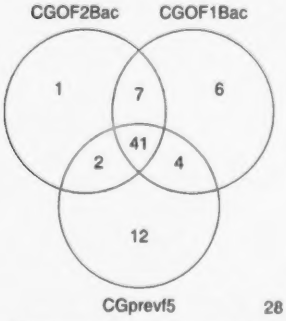


FIG. 2. Venn diagram for Canada goose fecal samples testing positive with the CGOF1-*Bac*, CGOF2-*Bac*, and/or CG-*Prev* f5 PCR assay. The number outside the circles indicates the number of Canada goose fecal samples for which none of the markers were detected.

geese, the concentrations of CGOF1-*Bac* and CGOF2-*Bac* were high, reaching levels up to 8.8 and 7.9 $\log_{10}$ copies g^{-1}, respectively (Fig. 1).

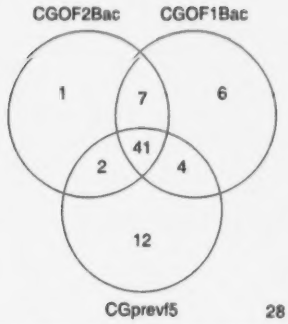
The potential of the Canada goose-specific *Bacteroides* qPCR assays to detect Canada goose fecal pollution in an environmental context was tested using water samples collected weekly during September to November 2009 from 8 shoreline sampling sites at Wascana Lake (see File S1 and Fig. S1 in the supplemental material). Wascana Lake is an urban lake, located in the center of Regina, that is routinely frequented by Canada geese. In brief, a single water sample of approximately 1 liter was taken from the surface water at each sampling site. Each water sample was analyzed for *Escherichia coli* enumeration using the Colilert-18/Quanti-Tray detection system (IDEXX Laboratories, Westbrook, ME) (8) and subjected to DNA extraction (with a PowerSoil DNA extraction kit [Mo Bio Inc., Carlsbad, CA]) for the detection of *Bacteroidales* 16S rRNA genetic markers using the *Bacteroidales* order-specific (All-*Bac*) qPCR assay (14), the two Canada goose-specific (CGOF1-*Bac* and CGOF2-*Bac*) qPCR assays developed in this study, and the human-specific (BacH) qPCR assay (17). All real-time and conventional PCR procedures as well as subsequent data analysis are described in the supplemental material and methods. The *E. coli* and All-*Bac* quantification data demonstrated that Wascana Lake was regularly subjected to some form of fecal pollution (Table 2). The All-*Bac* genetic marker was consistently detected in high concentrations (6 to 7 $\log_{10}$ copies 100 ml^{-1}) in all the water samples, while *E. coli* concentrations fluctuated according to the sampling dates and sites, ranging from 0 to a most probable number (MPN) of more than 2,000 100 ml^{-1}. High concentrations of *E. coli* were consistently observed when near-shore water experienced strong wave action under windy conditions or when dense communities of birds were present at a given site and time point.

The frequent detection of the genetic markers CGOF1-*Bac* (in 65 of 75 water samples [87%]), CGOF2-*Bac* (in 55 of 75 samples [73%]), and CG-*Prev* f5 (in 60 of 75 samples [79%]) and the infrequent detection of the human-specific *Bacteroidales* 16S rRNA gene marker BacH (17) (in 5 of 75 water samples [7%]) confirmed that Canada geese significantly contributed to the fecal pollution in Wascana Lake during the

TABLE 2. Levels of *E. coli* and incidences of the Canada goose-specific (CGOF1-*Bac* and CGOF2-*Bac*), human-specific (BacH), and generic (All-*Bac*) *Bacteroidales* 16S rRNA markers at the different Wascana Lake sites sampled weekly[a]

Site	*E. coli*			All-*Bac*			CGOF1-*Bac*			CGOF2-*Bac*			BacH		
	No. of positive water samples/ total no. of samples analyzed (%)	Min level–max level (MPN $100\ ml^{-1}$)	Mean level (MPN $100\ ml^{-1}$)	No. of positive water samples/ total no. of samples analyzed (%)	Min level–max level (log copies $100\ ml^{-1}$)	Mean level (log copies $100\ ml^{-1}$)	No. of positive water samples/ total no. of samples analyzed (%)	Min level–max level (log copies $100\ ml^{-1}$)	Mean level (log copies $100\ ml^{-1}$)	No. of positive water samples/ total no. of samples analyzed (%)	Min level–max level (log copies $100\ ml^{-1}$)	Mean level (log copies $100\ ml^{-1}$)	No. of positive water samples/ total no. of samples analyzed	Min level–max level (log copies $100\ ml^{-1}$)	Mean level (log copies $100\ ml^{-1}$)
W1	8/8 (100)	6–196	71.1	8/8 (100)	6.2–8.1	6.9	6/8 (75)	0–4.7	2.4	4/8 (50)	0–4	1.7	2/8	0–3.7	1.7
W2	9/10 (90)	0–1,120	194	10/10 (100)	5.8–6.8	6.4	9/10 (90)	0–3.7	2.6	8/10 (80)	0–3.3	2.2	0/10	0	0
W3	10/10 (100)	6–1,550	534	10/10 (100)	6–7.8	7	10/10 (100)	2.9–4.8	3.8	10/10 (100)	2–4.5	3.4	0/10	0	0
W4	10/10 (100)	16–1,732	529	10/10 (100)	6.4–7.6	7	10/10 (100)	3.2–4.6	3.9	10/10 (100)	2.8–4.3	3.4	0/10	0	0
W5	10/10 (100)	2–2,420	687	10/10 (100)	5.5–6.9	6.3	7/10 (70)	0–3.2	1.7	5/10 (50)	0–3.1	1.2	0/10	0	0
W6	10/10 (100)	3–1,990	389	10/10 (100)	5.5–7	6.3	9/10 (90)	0–4.3	2.8	6/10 (60)	0–5.1	2	1/10	0–3.4	1.3
W7	7/7 (100)	5–2,420	445	7/7 (100)	5.7–7.8	7	6/7 (86)	0–3.8	2.6	5/7 (71)	0–4.4	2.4	2/7	0–5.1	2.8
W8	10/10 (100)	17–980	160	10/10 (100)	6.3–8.6	7.1	8/10 (80)	0–4.6	2.8	7/10 (70)	0–4.4	2.3	0/10	0	0

[a] Min, minimum; max, maximum.



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[a] Min, minimum; max, maximum.

sampling period. Highest mean concentrations of both CGOF1-*Bac* and CGOF2-*Bac* markers were obtained at the sampling sites W3 (3.8 and 3.9 $\log_{10}$ copies 100 ml^{-1}) and W4 (3.4 $\log_{10}$ copies 100 ml^{-1} for both), which are heavily frequented by Canada geese (Table 2), further confirming their significant contribution to fecal pollution at these particular sites. It is worth noting that concentrations of the CGOF1-*Bac* and CGOF2-*Bac* markers in water samples displayed a significant positive relationship with each other (correlation coefficient = 0.87; $P < 0.0001$), supporting the accuracy of both assays for identifying Canada goose-associated fecal pollution in freshwater.

In conclusion, the CGOF1-*Bac* and CGOF2-*Bac* qPCR assays developed in this study are efficient tools for estimating freshwater fecal inputs from Canada goose populations. Preliminary results obtained during the course of the present study also confirmed that Canada geese can serve as reservoirs of *Salmonella* and *Campylobacter* species (see Fig. S3 in the supplemental material). Therefore, future work will investigate the cooccurence of these enteric pathogens with the Canada goose fecal markers in the environment.

Nucleotide sequence accession numbers. The *Bacteroidales* 16S rRNA gene sequences have been submitted to GenBank under accession numbers from GU222166 (clone CGOF1) to GU222217 (clone CGOF52).

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